UNIVERSIDAD DE SANTIAGO DE COMPOSTELA
FACULTAD DE VETERINARIA
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Caracterización y comparación de las señales de transducción implicadas en la activación de las células HMC-1^{560} y HMC-$1^{560,816}$

Tesis doctoral

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INFORMA

Que la tesis doctoral titulada: **"Caracterización y comparación de las señales de transducción implicadas en la activación de las células HMC-1^{560} y HMC-1^{560,816}"**, recogida en la presente memoria, de la que es autora la Licenciada en Farmacia **Kristin Löber**, ha sido realizada bajo su codirección y cumple las condiciones exigidas para que su autora puede optar al grado de Doctora por la Universidad de Santiago de Compostela, otorgando si aprobación para la lectura y defensa de la misma.

Para que así conste a los efectos oportunos, firma la presente en Lugo, a 11 de Diciembre de 2008

Fdo: Luis M. Botana López Fdo: Kristin Löber

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ABREVIATURAS

AE	Transportadores de Cl^-/HCO_3^- independientes de Na^+
ASM	Mastocitosis sistémica agresiva
Asp	Aspartato
ATP	Trifosfato de adenosina
BTR	Transportadores de Cl^-/HCO_3^- dependientes de Na^+
CA	Anhidrasas de carbono
CM	Mastocitosis cutánea
COX	Ciclooxigenasa
CTMC	Connective tissue mast cells
DAG	Diacilglicerol
DIDS	Ácido 4,4'-diisotiocianostilbeno-2,2'-disulfónico
ERK	Cinasa regulada por señales extracelulares
GM-CSF	Factor estímulador de colonias de granulocitos y macrófagos
Gly	Glicina
HMC-1	Human Mast Cell Line
IFN	Interferon
IgE	Inmunoglobulina E
IL	Interleucina
IP_2	Fosfatidil inositol 4,5 bisfosfato
IP_3	Inositol 1,4,5-trifosfato
JAK	Janus tirosina cinasas
LT	Leucotrieno
LOX	Lipoxigenasa
MAPK	Cinasa activada por mitógeno

µm	Micrometro
mM	Milimolar
MMC	Mucosal mast cells
nM	Nanomolar
NBC	Cotransportadores de Na^+/HCO_3^-
NCBE	Transportadores de Cl^-/HCO_3^- dependientes de Na^+
NHE	Transportadores de Na^+/H^+
NGF	Nerve growth factor
PG	Prostaglandina
pH_i	pH intracelular
PI3K	Cinasa fosfatidilinositol-3
pg	picogramo
PKB	Proteina cinasa B
PKC	Proteina cinasa C
PLC	Fosfolipasa C
PMA	Forbol-12-miristato-13-acetato
SERCA	ATPasa de Ca^{2+} del retículo endoplásmico
SCF	Stem cell factor
SLC4	Transportadores solubles 4
SM	Mastocitosis sistémica
STAT	Signal transducers and activators of transcription
TNF-α	Factor necrótico tumoral α
TXA	Tromboxano
TyrK	Tirosina cinasa
Val	Valina
WHO	World Health Organisation

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1. INTRODUCCIÓN

1.1 Mastocitos

1.1.1 Morfología, fenotipo, origen y distibución

Los mastocitos son células mononucleadas, no excitables de forma redonda o alargada que contienen gránulos. El mastocito humano fue descrito por primera vez por Paul Ehrlich en el año 1878 quien en experimentos con colorantes del grupo de las anilinas observó un cambio de color de azul a violeta por la gran cantidad de gránulos en el citoplasma de estas células. La alta densidad de estos gránulos alrededor del núcleo le dio la impresión de células cebadas por lo que acabo llamándolas "Mastzellen" [57, 59, 58].

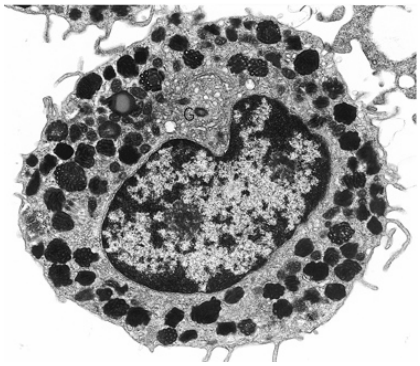


Foto 1: Mastocito (http://www.sciencefriday.com/newsbriefs/read/100)

Aunque los mastocitos tienen muchas características en común, no representan una población homogénea, lo cual es un punto importante en la investigación de sus funciones. Las primeras diferencias fenotípicas se han observado en mastocitos de roedor y se han clasificado en mastocitos del tejido conectivo (connective tissue mast cells = CTMC), que aparecen en la piel y la cavidad del peritoneo, y mastocitos de la mucosa (mucosal mast cells = MMC), que se encuentra en la lamina intestinal propria. Las diferencias entre las dos poblaciones incluyen el tamaño, el contenido de los gránulos y su función y sus reacciones frente a estímulos e inhibidores [3, 19, 60, 182]. Los CTMC tienen un tamaño de 10–20 µm. Contienen entre 10–20 pg de histamina y entre 1

y 2 pg de serotonina por célula. En cambio los MMC son más pequeños entre 5 y 10 µm y contienen solo aproximadamente 1 pg de histamina y menos de 0,5 pg de serotonina por célula. Mientras ambas poblaciones pueden estar activadas tras la agregación del receptor antigenico FcεRI, solamente las células CTMC están activadas tras la estimulación con el compuesto 48/80 y la sustancia P. Además el cromoglicato de sodio solo inhibe la desgranulación de las células CTMC. Por todas sus características, las CTMC también se denominan como mastocitos típicos mientras que los MMC se nombran como mastocitos atípicos [137].

Los mastocitos humanos también exhiben diferencias en la morfología, la estructura, el contenido de los gránulos y en la sensibilidad frente a distintos estímulos [52, 142, 151, 177]. Las poblaciones se han clasificado según el contenido citoplasmático de los gránulos en proteasas neutras [99]. La base de la clasificación es el hecho de que los dos tipos de mastocitos, MC_{TC} y MC_T, con distinta expresión de proteasas, aparecen en diferentes tejidos. Las células MC_{TC} contienen triptasa, químasa, carboxipeptidasa y catepsina G y forman el 99% de los mastocitos de la piel. Los MC_T solo expresan triptasa y forman menos del 1% de los mastocitos de la piel, pero el 93% del tejido alveolar [100]. Otro foco interesante de la distribución de los dos fenotipos es el intestino delgado. Mientras que en la mucosa dominan las células MC_T (81%), en la submucosa destacan los MC_{TC} (77%) [137]. Debido a sus distintos contenidos de proteasas los gránulos de los MC_T se disponen de forma enrollada mientras que los de los MC_{TC} se disponen en forma de reja [50, 51]. Teniendo en cuenta las funciones con respecto al contenido de proteasas, se ha demostrado que el numero de los MC_T, pero no de los MC_{TC}, está reducido en pacientes inmunodeficientes, considerando que la generación y el mantenimiento de una población normal de células MC_T es dependiente de las células T [101]. Además se ha observado que el cromoglicato de sodio solo inhibe los MC_T [41, 149, 151]. En muchos aspectos parece que las células MC_{TC} son similares a los CTMC de roedores y los MC_T a los MMC. Sin embargo no se pueden

prever todos las características de los mastocitos humanos enfrente de las células de rata y ratón.

	MC_{TC}	MC_T
Mediadores		
Histamina, Heparina	+	+
Triptasa	+	+
Quimasa	+	+
Distribución		
Piel	+++	(+)
Submucosa intestinal	+++	++
Bronquios	+++	++
Pulmón	+	+++
Tejido alveolar	(+)	+++

Tabla 1: Diferencias entre los dos fenotipos de mastocitos humanos

A partir de numerosos estudios se ha demostrado que los mastocitos humanos derivan de células precursoras hematopoyéticas indiferenciadas de la medula ósea. Estas células pluripotentes expresan FcεRI y FcγRII/III tempranamente durante su desarrollo aunque todavía no exhiben una maduración total de los gránulos y de otras características morfológicas. Diferentes citocinas como IL-3, SCF (Stem Cell Factor), IL-4, IL-9, IL-10 o NGF afectan al crecimiento y la diferenciación de los mastocitos [18, 81, 93, 98, 187, 194]. Las células precursoras penetran, todavía indiferenciadas, en la circulación y desde ahi van a los tejidos periféricos en donde desarrollan su fenotipo final con influencia de SCF y otras citocinas locales [76, 109].

1.1.2 Mediadores liberados

Los mediadores producidos por los mastocitos pueden ser clasificados en tres grupos:

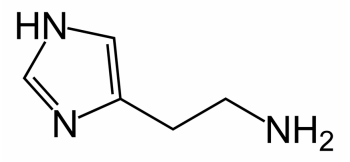
1) mediadores preformados asociados con los gránulos
2) mediadores sintetizados *de novo* a partir de los lípidos
3) citocinas.

1.1.2.1 Mediadores preformados asociados con los gránulos

Los mediadores preformados están compactados en los gránulos secretores y son liberados al medio extracelular rapidamente tras la activación de la célula. Entre estos mediadores destaca: histamina, proteasas de serina, carboxipeptidasa A y proteoglicanos (heparina, sulfato de condroitina E).

Histamina

La histamina es la única amina depositada en los mastocitos humanos, mientras que en otras especies, los gránulos contienen además otras aminas. Por ejemplo, los mastocitos de roedor almacenan también serotonina.



Formula 1: Histamina

Los mastocitos humanos aislados del pulmón, de la piel, de los tejidos linfáticos y del intestino delgado presentan un contenido de histamina de entre 3 y 8 pg por célula. En mastocitos de peritoneo de rata se encuentran entre 10 y 30 pg por célula, mientras que los de la mucosa contienen solo entre 1 y 3 pg por célula [66, 67, 175]. La síntesis de esta amina se lleva a cabo en el aparato de Golgi de los mastocitos y los

basófilos a partir del ámino ácido L-histidina tras su decarboxilación por la histidina decarboxilasa [212].
En los gránulos, la histamina está asociada con residuos ácidos de heparina y de sulfato de condroitina E y se excreta al medio extracelular mediante intercambio de cationes.

La histamina está implicada en varios procesos inmunologicos, fisiológicos y patológicos. Tras la unión con uno de sus receptores H1, H2, H3 o H4, que se encuentran específicamente expresados en las membranas de diferentes células, se desencadena una activación celular [92, 226, 227]. En los vasos sanguíneos la histamina provoca, por activación de los receptores H1, una vasodilatación y un aumento en la permeabilidad de los vasos. La dilatación de estos vasos lleva a una caída de la tensión y la producción de adrenalina. A parte de eso aparecen edemas en la piel y la mucosa que se conoce como síntomas típicos de alergias, asi como el enrojecimiento y el picor. En los bronquios la histamina induce, mediante la activación de los receptores H1, una bronchoconstricción que tiene papel patológico en el asma [165]. En la mucosa del estómago la amina estimula mediante los receptores H2 a las células parietales, lo que resulta un aumento de la secreción de ácido gástrico. En el sistema nervioso central la histamina actúa como neurotransmisor e influye mediante receptores presinápticos del tipo H3 la secreción de otros transmisores y la regulación de la activación de neuronas [144, 183]. Los receptores H4 están expresados en eosinófilos y mastocitos. Antagonistas de estos receptores han sido estudiados en modelos animales como sustancias antiinflamatorias [47, 148, 202].

Proteoglicanos

Los proteoglicanos pueden, por sus específicas características fisicoquímicas, actuar como mediadores extracelulares y también como ligante intracelular para otros mediadores preformados que podrían dañar la célula si están libres [137]. En los mastocitos humanos se han

encontrado los proteoglicanos heparina y sulfato de controitina E [138, 195]. Ambos proteoglicanos estabilizan las proteasas de los mastocitos e influyen en la actividad de otras enzimas. La heparina parece estar más expresada en los mastocitos del tejido conjuntivo que en los de la mucosa y actúa de forma extracelular como anticoagulante [49].

Proteasas neutras

Los mastocitos humanos contienen las mismas tres clases de proteasas que los de ratón: quimasa, triptasa y carboxipeptidasa A [176]. La expresión de las proteasas en los gránulos depende de la especie y del desarrollo y de la maduración de la propia célula.

Proteasa I o *quimasa* esta presente en el 85% de los mastocitos humanos de la piel y de la submucosa del intestino. Sin embargo no se ha encontrado la proteasa en la mucosa intestinal o en el pulmón [99]. La quimasa humana no está afectada por la heparina pero su actividad está inhibida con inhibidores de la serina como α_1-antiquimotripsina o α_2-macroglobulina [173]. La quimasa tiene una variedad de funciones biológicas como convertir angiotensina I y II, estimular la secreción del mucus, degradar neuropéptidos y convertir el precursor de IL-1β en su forma activa [143, 214]. En los mastocitos de rata la quimasa es una proteasa neutra que se encuentra en la piel, el músculo, el peritoneo y las cavidades de la pleura [217]. Se ha demostrado que la enzima tiene características parecidas a la α-quimostripsina que fracciona los ésteres y péptidos unidos a los aminoácidos aromáticos [137].

La *triptasa* es la proteasa predominante en los mastocitos humanos y aparece en todos los tipos. En el pulmón, la piel y el tracto gastrointestinal es la proteasa más expresada. En los gránulos la triptasa está ligada a la heparina y tras la estimulación es liberada en un complejo junto al proteoglicano [11]. Los inhibidores de esterasas de serina del plasma y de los tejidos no inhiben su actividad enzimática. La triptasa fracciona el fibrinógeno, activa la colagenasa e hidroliza además

neuropéptidos [80, 169]. Un aumento en la concentración de la triptasa en el suero es un criterio en el diagnóstico de las enfermedades de mastocitos conocidas como mastocitosis [179, 201].

La *carboxipeptidasa A* también está almacenada en los gránulos de los mastocitos en un complejo con proteoglicanos [178]. Es una enzima hidrolítica, que rompe uniones éster y péptidos de aminoácidos aromáticos, parecida a la quimasa. La carboxipeptidasa A de los mastocitos humanos está asociada con el tipo de mastocitos MC_{TC} [100].

1.1.2.2 Mediadores sintetizados *de novo* a partir de lípidos

Estos compuestos se forman a partir de los lípidos cuando la célula ha sido activado. Estas sustancias, en particular los metabolitos oxidativos del ácido araquidónico, tienen un gran impacto en la inflamación inmunológica. [89, 117, 158, 185].

Productos de la Ciclooxigenasa (COX)

Tras la activación inmunológica los mastocitos humanos generan prostaglandinas (PG) y tromboxanos (TXA) a partir de ácido araquidónico por acción de la COX [116, 118, 123]. La PGD_2 es un inhibidor de la agregación plaquetaria, de la relajación de la musculatura lisa, de la vasodilatación del riñón y de la reabsorción del agua. Por efecto del tromboxano y de la $PGF_2\alpha$ aumentan las contracciones bronquiales y la respuesta a otros inductores como alérgenos en pacientes con asma. Además la PGD_2 influye en la regulación de la temperatura corporal y del sueño. Se han observado niveles altos de PGD_2 en la orina de pacientes con mastocitosis.
La principal función biológica del TXA_2 es participar en la hemostasia, sobre todo en procesos de coagulación y agregación plaquetaria [144, 183].

Productos de la Lipoxigenasa (LOX)

Los productos de la oxidación del ácido araquidónico por la LOX son principalmente los leucotrienos. Los leucotrienos son potentes constrictores de la musculatura lisa asi como de las vías aéreas de los pulmones [44]. De esta manera, y promoviendo además la secreción de mucus, participan en el desarrollo de los síntomas del asma. Además los leucotrienos toman parte en procesos de inflamación crónica, aumentando la permeabilidad vascular y favoreciendo la generación de edemas [119-121, 123, 124].

En particular el leucotrieno B_4 (LTB_4) se ha descrito como activador quimiotáctico de neutrófilos y eosinófilos, además de modificar las funciones de los linfocitos [122].

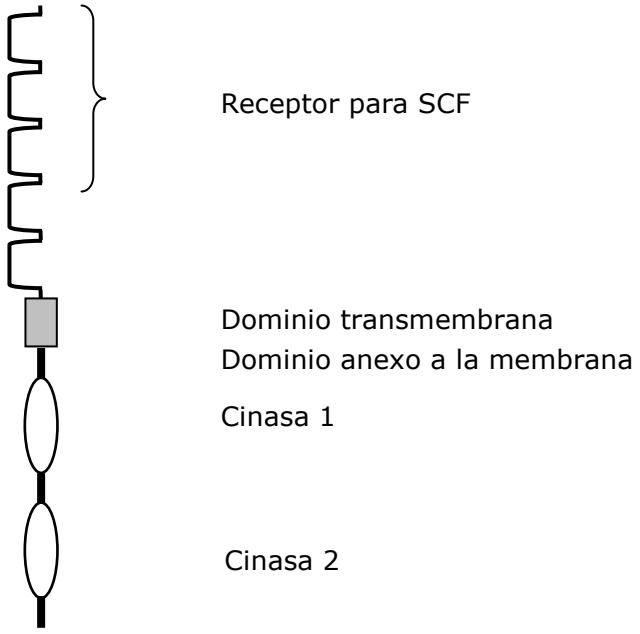
1.1.2.3 Citocinas

Las citocinas son proteínas o glicoproteínas sintetizadas y liberadas por células que regulan el crecimiento, la diferenciación o la respuesta inmunológica de otras células. Debido a sus funciones se pueden clasificar en cinco grupos: interferones, interleucinas, factores estimuladores de colonias, factor necrótico tumoral y quimiocinas. Los mastocitos son capaces de producir, en respuesta a su estimulación, diferentes citocinas. Entre ellas: IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, TNF-α, IFN-γ, GM-CSF y MCP-1 [31, 150]. Los mastocitos humanos expresan TNF-α, IL-4, IL-5, IL-6 y IL-8. IL-4 se encuentra principalmente en las células del tipo MC_{TC} mientras que IL-5 y IL-6 generalmente están presentes en las MC_T [24-28].

1.1.3 Activación e inhibición

1.1.3.1 Activación por c-kit

Los mastocitos expresan en sus membranas el receptor tirosina cinasa (TyrK) c-kit. El complejo es un miembro de la familia de las tirosina cinasas de transmembrana del tipo III y está compuesto por un dominio intracelular y otro extracelular [88, 125, 127]. En la parte extracelular está situado el receptor al cual se une el ligando natural, el SCF. Esta parte consta de cinco regiones parecidas a las que constituyen las inmunoglobulinas, tres de ellas están involucradas en la unión del ligando y del receptor. La parte intracelular consiste en un dominio anexo a la membrana y dos tirosina cinasas, separadas entre sí [125].



Esquema 1: Receptor tirosina cinasa c-kit

El factor del crecimiento de mastocitos SCF, es una citocina producida principalmente por las células del estroma. SCF se libera como un factor soluble del crecimiento o se expresa en la superficie de la membrana de las células del estroma [13, 62]. La unión del ligando con su receptor inicia la dimerización y fosforilación de c-kit y activa la TyrK intracelular que a su vez activa múltiples vías de transducción como las de la cinasa

fosfatidilinosiol-3 (PI3K), de los miembros de las Src cinasas, JAK/STAT y la cascada de las cinasas Ras-Raf-MAP. Las señales mediadas a través del receptor extracelular están implicadas en procesos muy importantes para la célula como proliferación, diferenciación, adhesión, migración, activación y supervivencia.

La ruta más estudiada tras la activación del c-kit es la de la *PI3K*. La cinasa es capaz de fosforilar fosfatidilinositoles como fosfatidilinositol-4-fosfato y fosfatidilinositol-4,5-bisfosfato. Se ha encontrado que uno de los productos de PI3K, el fosfatidilinositol-3,4-bisfosfato, juega un papel en la activación de la protein cinasa B (PKB), una cinasa serina-treonina involucrada en la supervivencia celular. El SCF induce la asociación de PI3K con c-kit y aumenta su fosforilación en tirosina en una de sus unidades que conduce en la activación de la síntesis de factores de la transcripción. Además se ha descrito que PI3K participa, tras la dimerización del c-kit, en la activación de las cinasas TEC y BTK que influyen, a través de la fosfolipasa C γ (PLCγ), en la señal de Ca^{2+} y así en la desgranulación de mastocitos [125, 192, 206].

Otra ruta inducida por la unión del c-kit con el SCF es la de la familia de las *Janus tirosina cinasas (JAK)*. La consecuencia de ello es la activación de las *STATs* (signal transducers and activators of transcription). La STAT es una familia de factores de la transcripción que asocia residuos forforilados en la tirosina de receptores activos para formar homo- y heterodímeros. Así se traslocan al núcleo celular, se unen al ADN y activan la transcripción de una variedad de genes. Por ejemplo, el SCF induce la asociación de STAT1 con c-kit y aumenta la fosforilación de la STAT1 en tirosina lo que incrementa su actividad al unirse al ADN. Se ha observado que la citocina también activa STAT5 e induce la fosforilación de STAT3 [125].

Miembros de la *familia Src* están involucrados en una amplia cadena de funciones celulares como son la adhesión, movilidad, progresión, diferenciación y viabilidad. La cinasa Lyn está expresada en altos niveles

en líneas celulares respondiendo al SCF y se une con el c-kit en el dominio anexo a la membrana. Además, se ha descrito que la citocina induce la asociación de Lyn con el c-kit y aumenta su activación. También Fyn, otro miembro de la familia Src se une al c-kit [125].

Tras la activación de varias cinasas, la dimerización del c-kit también induce la interacción de Ras con la cinasa de serina-treonina Raf, lo que lleva a la activación de MEK, una *MAP cinasa* (proteina cinasa activada por mitógeno). A continuación MEK fosforila otras MAP cinasas, las cuales al final de la cadena inducen la transcipción de varios genes [125].

Diferentes estudios han demostrado que la activación con SCF también puede influir en el proceso de la exocitosis de los mastocitos [45, 46, 206].

1.1.3.2 Activación inmunológica

La estimulación inmunológica de los mastocitos ocurre a través de la interacción de antígenos (alergeno) multivalentes con las inmunoglobulinas E (IgE). A través de las IgE el antígeno se une a una región Fc de receptores específicos de la membrana del mastocito, que se denominan FcεRI [155]. Cada uno de estos receptores es un tetrámero formado por una cadena α, una β y dos γ, que se encuentran unidas por puentes disulfuro. La subunidad α se encuentra unida a la β y ambas se unen después a la cadena γ. En la unidad α hay un sólo sitio de unión de alta afinidad para la IgE, que se encuentra fuertemente glicosilado. Los receptores FcεRI se encuentran distribuidos por toda la superficie celular del mastocito sensibilizado y un porcentaje de los mismos difunden libremente por la membrana [221].

Cuando un antígeno multivalente específico se une a dos o más moléculas de IgE tiene lugar la agregación de los receptores FcεRI y se inicia una cadena de fosforilación que afecta en primer lugar a la cinasas

de la familia Src. La tirosina Lyn fosforila a las subunidades β y γ del receptor. Este evento permite la unión y la fosforilación de la cinasa de tirosina Syk a los receptores agregados. A continuación Syk fosforila y activa la PLCγ1, que se transloca a la membrana, activándose la formación de inositol 1,4,5-trifosfato (IP_3) y diacilglicerol (DAG) y de este modo se activa la señal de Ca^{2+} y la proteina cinasa C (PKC) [172]. La agregación de los receptores FcεRI también puede estar inducida mediante otros estímulos [2, 14, 172]. Por ejemplo, las lectinas, como la concanavalina A, son capaces de unirse a las cadenas polisacarídicas de los receptores FcεRI, provocando su agregación y la consiguiente cascada intracelular, sin que haya tenido lugar una verdadera reacción antígeno-anticuerpo [159].

Además los mastocitos presentan en su membrana plasmática otros receptores, que les permiten responder a estímulos como adenosina y diversas citocinas [68, 197]. Se ha comprobado, que la adenosina potencia la respuesta a una estimulación inmunológica en mastocitos humanos de pulmón, así como en mastocitos peritoneales de rata, y mastocitos derivados de médula ósea de ratón [61].

1.1.3.3 Activación no inmunológica

Los mastocitos pueden responder también a un amplio número de estímulos no inmunológicos.

Así, la familia de moléculas polibásicas como el compuesto 48/80, mastoparan y polimixina B son capaces de estimular la exocitosis por los mismos mecanismos. Su efecto se basa en su estructura, que es la unidad catiónica justo al lado de una mitad hidrofóbica como una estructura espiral (mastoparan, sustancia P) o un anillo aromático (compuesto 48/80) [114]. En varios estudios se ha demostrado que la activación de los mastocitos con estas sustancias tiene lugar por proteinas G [152, 153, 207].

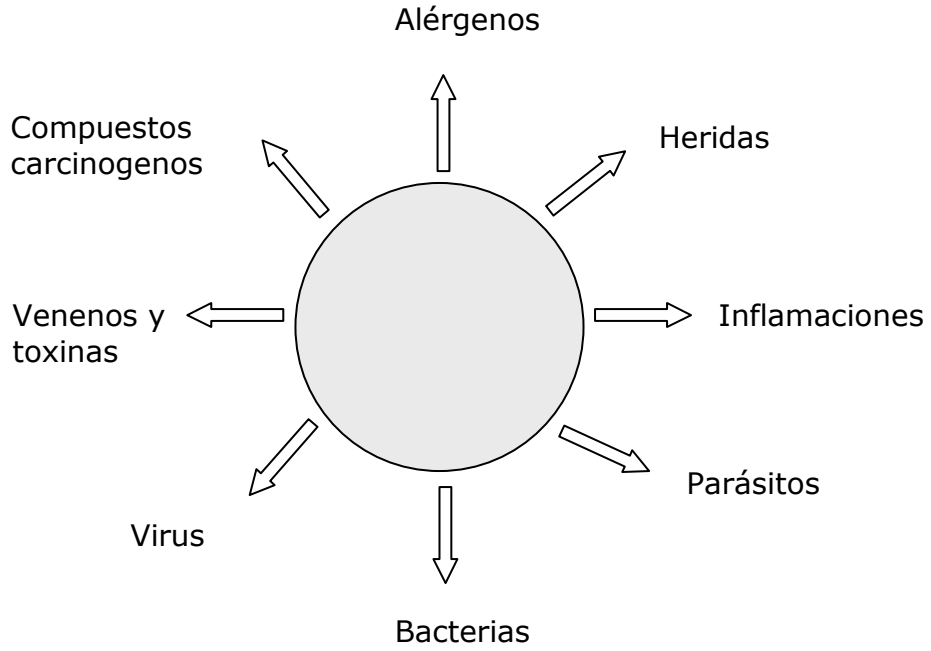
También una serie de péptidos inducen la liberación de histamina en los mastocitos. Se pueden distinguir tres grupos: péptidos correspondientes al dominio C_H4 de IgE, neuropéptidos y péptidos rab3A. Del primer grupo es la melitina, un veneno de abeja, y la hormona adrenocorticotrópica [103]. Se ha observado que fragmentos de estos compuestos corresponden a partes de la sequencia del dominio C_H4 de IgE humana [191]. Los péptidos sintetizados con esta misma secuencia también inducen la liberación de histamina en mastocitos peritoneales de rata. Los neuropéptidos como somatostatina o neurotensina así como el péptido Rab3A estimulan los mastocitos supuestamente por activación de proteinas G [137].

De la misma forma, también algunas citocinas, factores del complemento, dextranos y alcaloides activan los mastocitos de forma no inmunológica [137].

1.1.4 Funciones

Durante muchos años se consideró al mastocito como una célula perjudicial, debido a su destacada intervención en las reacciones alérgicas especialmente en procesos de tipo anafiláctico [75]. Esto ha cambiado al demostrar el papel clave de esta célula en la respuesta inmune frente a agresiones externas y en la inflamación. Además, el mastocito interviene en la curación de las heridas tras cortes o luz ultravioleta, y en la defensa frente a ciertos tumores [74, 91, 134, 141].
A parte de la activación inmunológica por IgE, se ha demostrado que los mastocitos están activados por una variedad de citocinas, péptidos, estímulos químicos y fisiológicos y sorprendentemente por un número extenso de sustancias biológicas como bacterias, productos de bacterias, virus, productos de la activación del complemento, venenos de animales y péptidos generados tras la infección con patógenos [130, 133, 135, 139, 140, 210, 215]. El hecho de que los mastocitos se encuentran en tejidos que forman la barrera entre el organismo y el exterior ha supuesto la extistencía de una relación entre las funciones del mastocito

y la defensa del organismo. Por ejemplo los mastocitos están localizados en la piel, en el tracto gastrointestinal, en el tracto pulmonar y también en las proximidades de las terminaciones nerviosas de intestino y vías respiratorias, así como en las meninges y en el cerebro [89, 90]. En los últimos años se ha demostrado que los mediadores de los mastocitos están involucrados en reclutar y activar monocitos, macrófagos, células T, neutrófilos, basófilos y eosinófilos. Además los mediadores están involucrados en la degradación de compuestos de venenos de serpiente y de otros mediadores tóxicos. También se han observado efectos en células epiteliales y dendríticas.



Esquema 2: Resumen sobre las funciones de los mastocitos

Por otra parte, los mastocitos pueden actuar como células presentadoras de antígeno, ya que expresan en su membrana celular el complejo mayor de histocompatibilidad de tipo II [69].

El hecho de que los mastocitos se localicen también en el sistema nervioso central se relaciona con un papel integrador de ciertos estímulos hormonales [184]. A pesar de que la relación funcional entre los mastocitos y el sistema nervioso no ofrece dudas, esta es aún relativamente poco conocida y no ha sido descrita en profundidad.

La implicación del mastocito en la inflamación incluye sobre todo las respuestas inducidas por los mediadores liberados. Entre estas respuestas destacan los efectos vasculares debidos a la histamina, el reclutamiento de células al tejido agredido y la estimulación de la proliferación y función leucocitaria por la liberación de citocinas. Los efectos pueden ser tanto pro- como anti-inflamatorios o solo inmunoregulatorios.

Hasta ahora no se ha encontrado ningún animal que presente una ausencia completa de mastocitos [137].

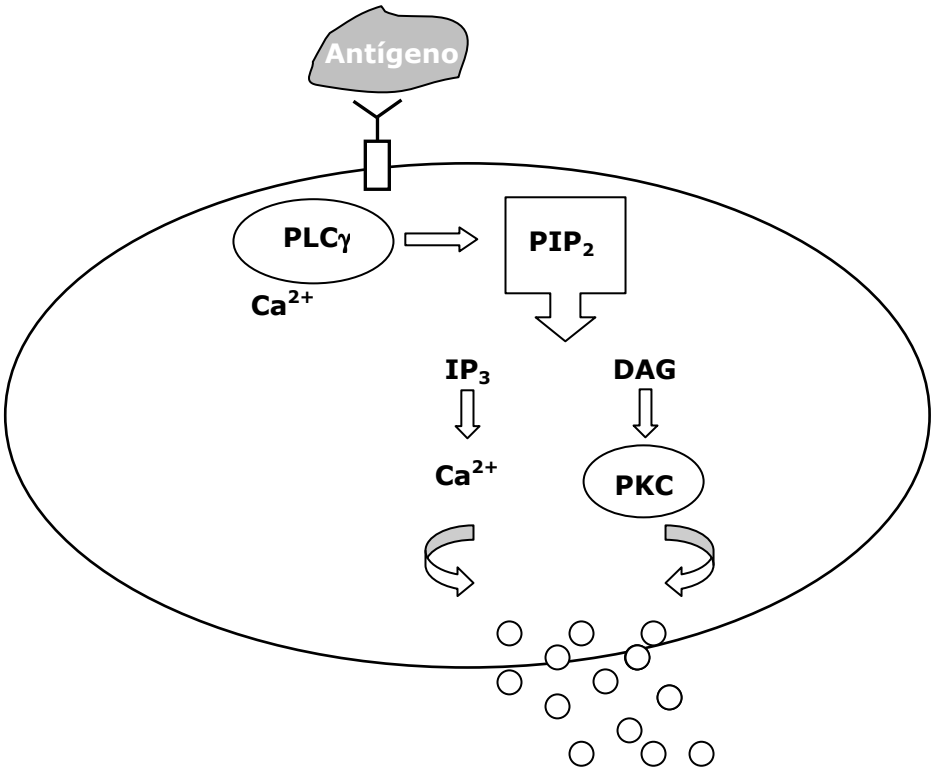
La estimulación de los mastocitos induce la activación de diferentes señales intracelulares como la de Ca^{2+} citosólico, de pH intracelular (pH_i) o de PKC [9].

1.2 Señal de Ca^{2+}

El Ca^{2+} actúa como segundo mensajero que interviene en la regulación de importantes procesos celulares como secreción, contracción, crecimiento y proliferación celular. Desde su descripción como una señal intracelular, se han publicado muchos estudios sobre la regulación de su concentración citoplasmática, donde se ubica la mayoría de enzimas y moléculas con las que interacciona. La concentración basal de Ca^{2+} en el citosol es aproximadamente de 100 nM mientras que en el medio extracelular es de 1 mM. Para mantener los niveles básales las células tienen diversos canales e intercambiadores que retiran el Ca^{2+} citosólico en exceso hacia el medio extracelular, o bien al interior de reservorios intracelulares, donde el ión permanece almacenado [225].

En general, en los mastocitos la subida en la concentración del Ca^{2+}, al igual que en otras células no excitables, es un proceso que transcurre en dos fases. Primero, se observa una elevación inicial debido al vaciamiento de los reservorios intracelulares de Ca^{2+}, a la que sigue una fase donde los niveles bajan lentamente en forma de meseta. El vaciamiento de los reservorios produce la señal para iniciar la segunda fase que corresponde a la entrada de Ca^{2+} desde el medio extracelular a través de la membrana plasmática [43]. El IP_3 está implicado en este proceso y en la movilización del Ca^{2+} citosólico. La unión de IP_3 a sus receptores, los cuales son canales de Ca^{2+} activados por ligandos que están en la membrana del retículo endoplásmico, aumenta la posibilidad de que se abran los canales de Ca^{2+} de la membrana y el ión entre al citosol. Fisiológicamente, la concentración citosólica de IP_3 es muy baja, pero sus niveles se incrementan rápidamente en respuesta a una estimulación de receptores de la membrana plasmática. En mastocitos, la estimulación puede tener lugar por dos vías que terminan en la fosforilación de fosfolipasas C (PLC): la agregación de FcεRI o la activación de proteinas G. La activación inmunológica por FcεRI resulta en la fosforilación de PLCγ1 en tirosina, que activa la enzima y se

transloca desde el citosol a la membrana. Ahí es donde hidroliza su sustrato, el fosfatidil inositol 4,5 bisfosfato (IP_2), que da lugar a la formación de IP_3 y DAG [10, 53]. La dependencia de este proceso del Ca^{2+} extracelular es única para la activación tras la agregación FcεRI [16, 17]. La activación de proteínas G activa PLCβ que también resulta en la formación de IP_3 y DAG. Los dos actúan como mensajeros segundarios. Mientras que el IP_3 moviliza el Ca^{2+} citosólico, el DAG forma parte en la activación de la mayoría de las PKC. El Ca^{2+} movilizado por el IP_3 y la PKC activada por el DAG estimulan simultáneamente la exocitosis de los mastocitos [147]. En este sentido se ha observado en mastocitos peritoneales de rata que el ionóforo del Ca^{2+} ionomicina, al igual que el IP_3, activa selectivamente una corriente del Ca^{2+} hacia adentro [97]. Se han realizado muchos estudios en mastocitos con este compuesto para determinar vías de tranducción moduladas por Ca^{2+}.



Esquema 3: La ruta de transducción tras la agregación FcεRI

En las células HMC-1^{560} se ha demostrado que una estimulación con ionomicina aumenta de manera dosis dependiente el Ca^{2+} citosólico por

el vaciamiento de los reservorios y también por la entrada del ión desde el medio extracelular. El ionóforo induce la liberación de histamina en estas células y una estimulación anterior de la PKC incrementa más este efecto de la ionomicina [156, 157].

Para mantener los niveles citosólicos de Ca^{2+} en torno a los valores básales, la célula dispone de un complejo sistema de canales e intercambiadores iónicos, que actúan tras una elevación en la concentración citosólica.

En la membrana plasmática de las células se encuentran las ATPasas de Ca^{2+} que son los sistemas más importantes para la salida de Ca^{2+} hacia el medio extracelular. Este transporte de Ca^{2+} está asociado a un transporte de H^+ en sentido opuesto y acoplado a la hidrólisis del trifosfato de adenosina (ATP) [35, 220]. Un segundo mecanismo encargado de la extrusión de Ca^{2+} hacia el exterior de la célula es el intercambiador Na^+/Ca^{2+}. Este sistema intercambia Ca^{2+} intracelular por Na^+ del medio extracelular, aprovechando el gradiente iónico. Sin embargo, cuando este gradiente de Na^+ se encuentra alterado, el intercambiador puede funcionar en modo reverso [29, 37, 65, 102, 112, 132].

Además, de mediante la expulsión de Ca^{2+} al medio extracelular, la célula puede regular la concentración citosólica de este ión mediante su entrada en diversos reservorios intracelulares [42, 72, 73, 224]. Uno de los principales es el retículo endoplásmico que puede actuar como fuente de Ca^{2+} a través de los receptores de IP_3 y de rianodina, que funcionan como canales de Ca^{2+}. Además, sirve para recoger el Ca^{2+} en en caso de una intensa subida del Ca^{2+} en el citosol a través de la membrana plasmática [82, 180]. La entrada del ión en este reservorio está mediada por las ATPasas de Ca^{2+} del retículo endoplásmico (SERCAs). Estas bombas median en el transporte de Ca^{2+} desde el citosol hasta el interior de los reservorios hidrolizando ATP.

Las mitocondrias son otro reservorio importante en el mantenimiento de los niveles citosólicos de Ca^{2+}. La entrada de Ca^{2+} en estos orgánulos transcurre a través de un transportador de baja afinidad que está situado en la membrana interna de las mismas. Dicho transportador está regulado mediante el potencial de la membrana, así como por una concentración citosólica de Ca^{2+} elevada. Debido a su baja afinidad, el transportador no es sensible a los incrementos fisiológicos del ión [21]. En general, las mitocondrias pueden captar el Ca^{2+} que liberan los demás reservorios o que entra desde el medio extracelular. Además, regulan de modo positivo la entrada del ión a través de canales de la membrana plasmática, ya que al secuestrar el Ca^{2+} están impidiendo la retroalimentación negativa que este ión ejercería sobre dichos canales [95, 96].

En las células HMC-1^{560} se ha observado que el inhibidor de las ATPasas del Ca^{2+}, tapsigargina, es capaz de vaciar los reservorios intracelulares y activar la entrada de Ca^{2+} desde el medio extracelular. Sin embargo el compuesto por si solo no induce la liberación de histamina pero aumenta el efecto de la ionomicina sobre la exocitosis [156].

1.3 Señal de pH

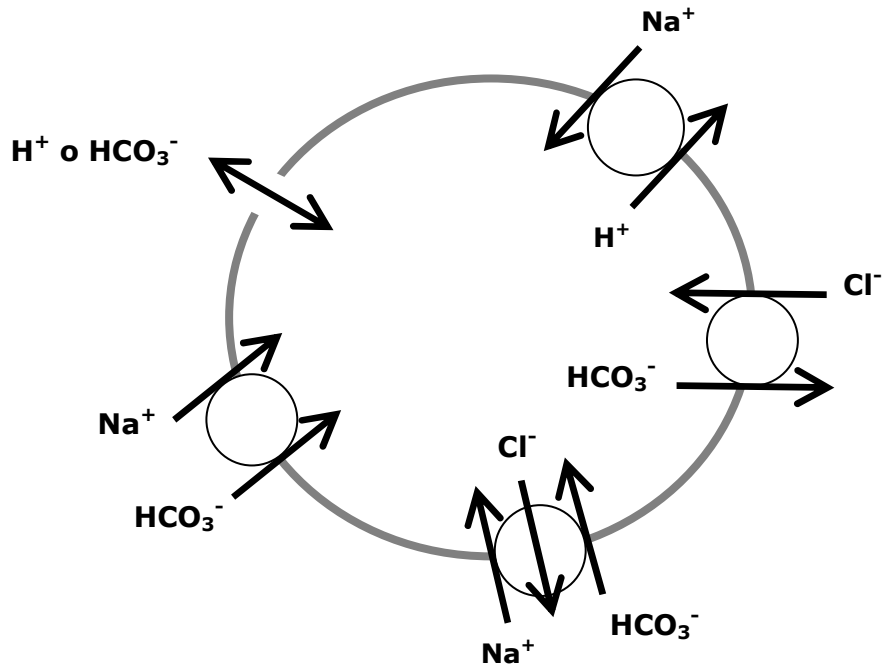
En mastocitos se ha estudiado diferentes mecanismos de la regulación del pH_i y apuntan la implicación del pH en el proceso de la exocitosis. Sobre todo muestran la participación del sistema de HCO_3^-/CO_2 y de la regulación de los protones [22, 104, 205, 204].

1.3.1 Transportadores de HCO_3^-

Uno de los mecanismos más importantes en la regulación del pH intracelular (pH_i) de células animales son los transportadores de HCO_3^-. Junto a la estabilización de pH_i basal juegan un papel en la división celular, la migración y el movimiento de otros iones por las membranas. Diferentes transportadores de HCO_3^- están expresados dependiendo de su función en distintos tejidos en donde actúan en un modo especializado [166, 167]. Todos forman parte de la familia de los transportadores solubles 4 (solute carrier) SLC4. Se distingue tres grupos de transportadores de HCO_3^- en función a los iones participantes: transportadores de Cl^-/HCO_3^- independientes de Na^+ (AE), cotransportadores de Na^+/HCO_3^- (NBC) y transportadores de Cl^-/HCO_3^- dependientes de Na^+ (NCBE, BTR1) [167].

Principalmente AEs están activados durante la alcalinización intracelular transportando HCO_3^- al medio extracelular a cambio de Cl^-. Se han identificado cuatro isoformas: AE1, AE2, AE3 y AE4 [167]. El AE1 (SLC4A1) está expresado en las células rojas de la sangre y en partes del riñon. El intercambiador está estructural y fisiológicamente bien estudiado; no es sensible a cambios del pH intracelular y tampoco a iones NH_4^+. En cambio el AE2 (SLC4A2), que está expresado en células no excitables, es muy sensible al pH_i. Curiosamente está activado por NH_4^+ y fuertemente inhibido por H^+. El AE3 (SLC4A3) se ha encontrado en tejidos de corazón y de cerebro. El AE4 (SLC4A9) está expresado en células de riñón. Por la homología en la secuencia de sus aminoácidos en

común con los NBCs, el intercambiador fue objeto de controversia por su dependencía del Na^+. AE1-AE3 son capaces de unirse a la anhidrasa de carbono (CA) II y IV y estimularla. Las CA catalizan la reacción CO_2 + H_2O <-> H^+ + HCO_3^- y afectan así el pH_i de algunas células.



Esquema 4: HCO_3^- y H^+ transportadores

Los cotransportadores Na^+/HCO_3^- transportan iones Na^+ y HCO_3^- desde el medio extracelular a través de la membrana independiente de Cl^- y alcalinizan así el pH_i de la célula. El NBCe1 (SLC4A4) es electrogénico y está en primer lugar expresado en el riñón, el páncreas, el hígado y el tracto gastrointestinal [131, 166, 174]. El NBCe2 (SLC4A5) se ha detectado en el hígado, el bazo y otros tejidos. El transportador media en el transporte de Na^+/HCO_3^- independiente de Cl^- y puede estar inhibido por el derivado de stilbeno DIDS (ácido 4,4'-diisotiocianostilbeno-2,2'-disulfónico) [171]. El NBCn1 (SLC4A7) es idéntico en un 59% al NBCe1 y se ha encontrado en la retina humana y en la médula espinal, el músculo, el timo, la tráquea y otros tejidos [39]. En estudios funcionales con oocitos se ha demostrado un transporte electroneutro de Na^+/HCO_3^- independiente de Cl^- insensible a DIDS y sensible a la amilorida. Otro estudio posterior con un homólogo NBCn1

de rata ha demostrado que es resistente a la amilorida pero parcialmente sensible al DIDS [39, 160, 161]. El NDCBE (SLC4A8) está expresado en los testículos, en el ovario, el cerebro, la médula ósea y otros tejidos. Es un 56% idéntico con NBCe1 y un 76% con el NBCn1. Ha sido muy discutido si el transportador electroneutro exige Cl^- para su funcionamiento. Por un lado un estudio ha demostrado que la disminución de Cl^- no afectó el cotransporte de Na^+/HCO_3^- mientras que por otro lado, oocitos que expresan NDCBE intercambian Cl^-/HCO_3^- de manera dependiente del Na^+ [12, 79]. Hasta ahora no existe mucha información sobre las últimas dos isoformas de la familia genética de intercambiadores de HCO_3^-. El NCBE (SLC4A10) es un transportador electroneutro de Na^+/HCO_3^- expresado en el cerebro, el riñón, el íleon y otro tejidos, que se puede inhibir con DIDS [55]. El BTR1 (SLC4A11) es un transportador de $Na^+/B(OH)_4^-$ que se han encontrado hace poco en el riñón humano [154].

1.3.2 Transportadores de Na^+/H^+

Los transportadores de Na^+/H^+ (NHE) fueron clonados por primera vez en 1989. Desde entonces se han encontrado 9 isoformas (NHE1-NHE9). NHE2 y NHE3 están localizados predominantemente en la membrana apical del epitelio y expresados en altos niveles en el riñón y en el intestino. NHE4 se encuentra principalmente en el estomago pero también en el riñón, el intestino, el útero, el cerebro y el músculo. NHE5 es predominante en el cerebro y en niveles bajos en otros tejidos no epiteliales. NHE6-NHE9 están presentes en compartimentos intracelulares para regular el pH luminal y la concentración de los cationes en los orgánulos. NHE7 se diferencia de las otras isoformas en translocar, además de Na^+, también K^+ en intercambio con H^+. La actividad de los NHEs está inhibida por la amilorida y sus derivados en el siguiente orden considerando la sensibilidad frente a estos compuestos: NHE1 >> NHE2 > NHE5 > NHE3 > NHE4 [63, 186].

La isoforma NHE1 está omnipresente en la membrana citoplasmática de casi todas las células y está involucrada en el crecimiento, la diferenciación, la migración, el flujo de Na^+ y el volumen celular. NHE1 consiste en 12 dominios de transmembrana y una cola hidrofílica a la cual se une una cadena de proteína cinasas y otras enzimas regulatorias. ERK vía MAPK, $p90^{rsk}$, NIK y CAMKII fosforilan y activan NHE directamente y también la PKC y la PKD aumentan la actividad de NHE pero no por fosforilación directa. El transportador cambia su dependencia al pH en presencia de moduladores de Ca^{2+} y la CAII. Mientras que la calmodulina, CHP y CAII incrementan su actividad, la tescalina la reduce. Otras moléculas que se unen a NHE son PIP2 o HSP70 [56, 63, 186].

En condiciones fisiológicas NHE1 está activo durante una acidificación intracelular e intercambia Na^+ desde el medio extracelular por H^+ del interior. Su activación puede llevar a una alcalinización intracelular que se ha demostrado como uno de los primeros eventos en la transformación de tumores [162]. En células transformadas o malignas, el NHE1 causa un cambio del gradiente del pH que resulta en la alcalinización del interior de la célula y la acidificación del medio extracelular [23]. Por el contrario, inhibidores de NHE1 inducen apoptosis en líneas tumorales de leucemia y de tumores mamarios [163, 164].

1.3.3 pH intracelular y mastocitos

El pH intracelular tiene un papel importante en la activación de los mastocitos y especialmente de las células HMC-1. Se ha demostrado en las células $HMC\text{-}1^{560}$ que la adición de NH_4Cl aumenta el pH_i de forma dosis dependiente sin provocar cambios en el Ca^{2+} citosólico. Este aumento es una señal suficiente para desencadenar el proceso de liberación de histamina. Además, la incubación simultanea con acido propionico muestra claramente una inhibición de forma dosis dependiente del aumento del pH y de la liberación de histamina. La exocitosis inducida por una alcalinización intracelular está modulada por

la PKC. Los activadores de estas enzimas incrementan la liberación de histamina aumentando aún más el pH_i [156, 157].

En mastocitos peritoneales de rata se ha observado que el pH_i desciende en un medio extracelular sin HCO_3^-. Retirando el Cl^- del medio externo se invierte el intercambio de Cl^-/HCO_3^- y se alcaliniza el interior de la célula. Alcalinización y acidificación se han demostrado como mecanismos independientes del Na^+. La alcalinización y su recuperación son inhibidas por DIDS y completamente dependientes de la presencia de HCO_3^- [104]. El mismo estudio muestra que el HCO_3^- disminuye la liberación de histamina por cambios en el transporte de iones y no debido al pH_i. Otro estudio muestra que la presencia de HCO_3^- influye en la liberación de histamina dependiente del estímulo. En un medio con HCO_3^- la exocitosis inducida por PMA/ionóforo está reducida mientras que la liberación de histamina está aumentada si el estímulo es la tapsigargina [203, 205]. Además se ha observado que el pH_i de mastocitos de rata se alcaliniza en un medio sin HCO_3^- al añadir $NaHCO_3$. El efecto es menor con $KHCO_3$ y puede ser inhibido con DIDS y amilorida. Se concluye que la alcalinización en estas células está controlada por AEs y NHEs [204].

También el transportador de Na^+/H^+ tiene un papel importante en los mastocitos, por un lado para la entrada de Na^+ y por otro en la regulación del pH_i. Se ha demostrado en mastocitos de rata que la recuperación del pH_i tras una acidificación es dos veces más lento cuando las células están tratadas con amilorida [22, 33]. Además, mientras que la supresión de Ca^{2+} no afecta a la actividad de NHE, el inhibidor de las ATPasas de Ca^{2+} thapsigargina y la estimulación de las PKC por PMA activan el intercambiador [5]. Desde que se ha demostrado en mastocitos de rata que una alcalinización con NH_4Cl por si sola, sin cambiar niveles del Ca^{2+} intracelular, es capaz de inducir exocitosis en estas células, varias rutas de transducción han sido estudiadas con el compuesto en estas células [7, 8].

1.4 Proteina cinasas C (PKC)

Las PKC son cinasas de serina/treonina que fosforilan y activan una variedad de enzimas, que tienen un papel fundamental en el control de numerosos procesos celulares, como la regulación del metabolismo, las señales de transducción dependientes de receptores, el crecimiento y la diferenciación celular, la promoción de tumores, así como procesos de apoptosis y de secreción de neurotransmisores y hormonas. La familia de las PKC consiste en al menos 11 isoenzimas relacionadas estructuralmente, pero distintas en cuanto a su expresión en los diversos tejidos, así como en su regulación y sustratos [86]. Cada isoenzima está formada por una cadena polipeptídica que presenta un dominio regulador en el extremo amino y uno catalítico en el extremo carboxilo. El dominio regulador contiene las secuencias de unión a los fosfolípidos, que son necesarias para su activación, y la de otras moléculas reguladoras. Además, participa en las interacciones proteína-proteína que regulan la localización y actividad de las PKC. Por su parte, el dominio catalítico presenta actividad cinasa e incluye secuencias de unión al ATP y al sustrato [168].

Las distintas isoenzimas de este grupo se clasifican en función de su sensibilidad al Ca^{2+} y de si son activadas por DAG. Así, las isoenzimas α, βI, βII y γ son dependientes de Ca^{2+}, mientras que las isoformas δ, ε, η, μ y θ son independientes de este ión. Ambos grupos son activados por DAG, fosfatidilserina, ácidos grasos insaturados y el forbol-12-miristato-13-acetato (PMA) [145]. El tercer grupo comprende las isoformas atípicas ζ, λ y ι, cuya actividad es independiente de Ca^{2+} y de DAG [48]. En el caso de las isoenzimas activadas por DAG, la unión de esta molécula a la PKC provoca su activación y la translocación a la membrana. El PMA activa la PKC al unirse al mismo sitio que el DAG y por eso el compuesto es utilizado en estudios para simular el efecto del segundo mensajero. Sin embargo, el DAG puede ser metabolizado,

mientras que el PMA no se metaboliza, por lo que su efecto es mucho más duradero.

En mastocitos se ha observado que las PKCs están involucradas en la señal de transducción que da lugar a la exocitosis actúando en su mayoria favoreciendo a la desgranulación en respuesta a estímulos como IgE, ionóforos de calcio, NGF, tapsigargina, fluoruro sódico, e incluso es capaz de inducirla por sí misma [6, 22, 36, 87, 106, 211]. Por el contrario, se ha descrito un cierto efecto inhibidor sobre la estimulación inducida por el compuesto 48/80 [22]. Diversos esudios han mostrado que la PKCδ es un paso clave en el proceso que conduce a la exocitosis y que esta isoforma puede acutar como regulador negativo de este proceso tras estimulación con antigenos [38, 170, 188, 203]. PKCε está expresando en altos niveles en mastocitos y la agregación del receptor FcεRI la activa. Sin embargo, no se observan diferencias de la activación de proteinas importantes que conducen a la desgranulación entre mastocitos de ratones expresando PKCε o no [115]. En cambio, la exocitosis y la producción de IL-6 están inhibidas en ratones negativos en PKCβ [146]. Finalmente también la PKCθ participa en el proceso de la desgranulación [126]. Se ha descrito que la isoforma está activada tras la agregación FcεRI y además su inhibición con flavonol inhibe la entrada de Ca^{2+} y la liberación de mediadores [107]. Hay que tener en cuenta que los mostocitos son células heterogéneas y no todos expresan todas las isoformas de PKC y así su regulación y sus efectos pueden ser diferentes debido a la expresión y activación de otras enzimas [108]. Además en los mastocitos, las PKCs partipan en muchos procesos como la producción de citocinas, la regulación de la expresión de c-kit, la adhesión a distintas superficies o la regulación del pH por NHE [4, 15, 113, 192, 223].

1.5 Human Mast Cell Line HMC-1 (HMC-1)

1.5.1 Morfología, fenotipo, origen y distribución

Los mastocitos humanos HMC-1 fueron obtenidos de un paciente con leucemia mastocítica. De la línea HMC-1 se han cultivado dos sublíneas HMC-1^{560} y HMC-$1^{560,816}$ con distintos fenotipos y diferentes mutaciones en el c-kit [32, 192].

En las células HMC-1, el receptor tirosina cinasa c-kit está activo permanentemente por mutaciones en el proto-oncogen del c-kit, que cifra la proteína. Una estimulación SCF aumenta la fosforilación de c-kit aun más. Dichas mutaciones resultan en un cambio de aminoácidos en la proteína, por un lado Gly-560 -> Val (HMC-1^{560}) y por otro Asp-816 -> Val (HMC-$1^{560,816}$). El cambio en la posición 560 ocurre en el dominio anexo a la membrana e implica la activación del c-kit independientemente del ligando en las dos líneas. Esta mutación se encuentra a menudo en varias enfermedades malignas como el tumor gastrointestinal del estroma y también en la mastocitosis cutánea, que aparece casi siempre en niños [181]. Las células HMC-$1^{560,816}$ tienen las dos mutaciones. El cambio en la posición 816 ocurre en el parte intracelular de c-kit y cambia la conformación de la TyrK [1, 128, 222]. Esta mutación se encuentra en las células de más del 80% de los pacientes con mastocitosis sistémica y es un criterio en el diagnóstico de esta enfermedad [77, 200].

Aparte de su proliferación independiente del SCF, las células HMC-1 tienen mucho en común con los mastocitos tisuales, como la expresión de histamina, heparina y antígenos de la superficie. La mayor diferencia entre estas células y los mastocitos normales es la falta de la expresión de receptores FcεRI en las células HMC-1 [32]. Sin embargo debido a sus ventajas como cultivo celular se han realizado muchos estudios sobre la biología y las funciones de los mastocitos con la línea HMC-1.

Poca información está publicada sobre las dos sublíneas HMC-1^{560} y HMC-$1^{560,816}$ y sobre las consecuencias que tienen las respectivas mutaciones en la activación celular. Se ha observado que las células HMC-1^{560} son heterogéneas en su tamaño y crecen juntas agregándose unas a las otras, mientras que las HMC-$1^{560,816}$ son más pequeñas, homogéneas y crecen en una suspensión de células singulares. Las células con dos mutaciones crecen tras 7 dias tres veces más que las otras [192]. En las fotos 2 y 3 se muestran células HMC-1^{560} y HMC-$1^{560,816}$ pegadas a portaobjetos con polilisina en un microcopio confocal.

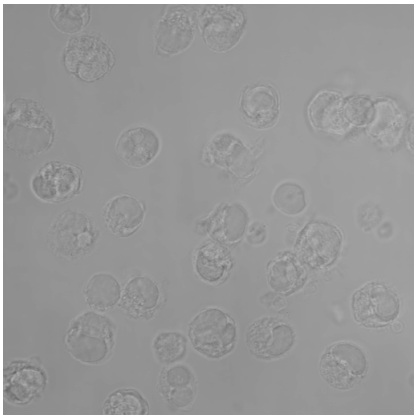


Foto 2: HMC-1^{560}

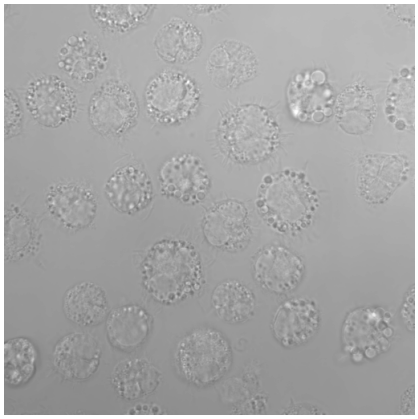


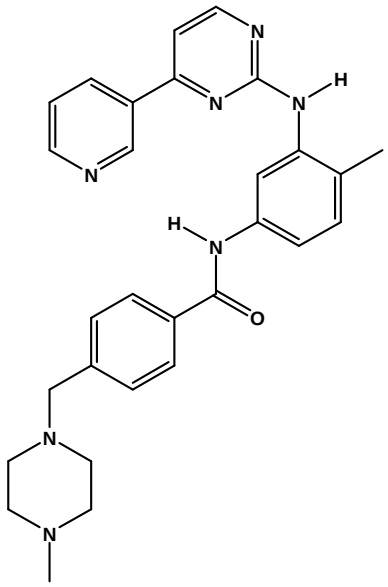
Foto 3: HMC-$1^{560,816}$

Ambas sublíneas expresan en sus superficies los antígenos CD13, CD32, CD44, CD54 y CD117 (c-Kit), pero son negativas para la expresión de la cadena α del complejo del receptor Fc e inmunoglobulina E (IgE). Además se ha visto que las células HMC-1^{560}, pero no las HMC-$1^{560,816}$ se pegan a superficies tratadas con colágeno I y IV, mientras que las dos líneas se adhieren a superficies con fibronectina y laminina. Después de la activación de PKC con PMA solo las células HMC-1^{560} pero no las HMC-$1^{560,816}$ se unen a vitronectina y tenascina [192].

1.5.2 Activación e inhibición por c-kit

En las dos líneas, HMC-1^{560} y HMC-$1^{560,816}$, se ha demostrado que la fosforilación constante del c-kit induce la proliferación de estas células independientemente del factor de crecimiento de mastocitos. Como se ha descrito anteriormente la activación del c-Kit pone en acción vías de transducción siguientes que acaban en la estimulación de factores de la transcripción. Se ha encontrado que el c-Kit está conectado con una de las subunidades de PI3K independientemente de la presencia del SCF. Además un tratamiento con el inhibidor Ly2940002 de PI3K inhibe la proliferación e induce apoptosis en las células HMC-1^{560} y HMC-$1^{560,816}$. Sin embargo no se han observado altos niveles de PKB forsforilado, pero el tratamiento del SCF induce la fosforilación de la proteína. Lo mismo pasa con ERK, una cinasa de la ruta de transducción de las cinasas Ras-Raf-MEK. Las células no estimuladas exhiben poca fosforilación en ERK, principalmente en ERK2, mientras que la estimulación con SCF fosforila ERK1 y ERK2 fuertemente. También las cinasas JAK/STAT y Scr están asociadas con el c-kit y se supone que están involucradas en la supervivencia y la proliferación de estas células [125, 192].

El inhibidor del receptor tirosina cinasa c-kit STI571, conocido como imatinib o Glivec®, se descubrió mientras se hacían pruebas de varios compuestos para la inhibición de la PKC [30]. El desarrollo de numerosos análogos químicos resultó en STI571, que inhibe el receptor del factor de crecimiento derivado de plaquetas y las tirosina cinasas de v-Abl, c-Abl, Bcr-Abl y c-kit.



Formula 2: STI571 (Glivec®)

El compuesto ha sido muy eficaz en mastocitos con el c-kit salvaje y aun más en células con la mutación 560. Por el mismo motivo ha sido muy eficaz en pacientes con tumor gastrointestinal del estroma. El fármaco se une con alta afinidad a la conformación inactiva de la cinasa intracelular del receptor y la deja en esta condición. STI571 actúa como un inhibidor competitivo del ATP en su sitio particular [181]. De esta modo es capaz de inhibir la proliferación e inducir la apoptosis de las células activadas permanentemente [88]. Sin embargo, el compuesto es ineficaz en células con la mutación 816, debido al cambio de la conformación que evita la unión de STI571 con el receptor de la TyrK [128]. De todas formas la efectividad del fármaco en el tratamiento de las mastoscitosis, tanto las sistémicas desarrolladas en adultos, como las cutáneas en niños, es muy discutida.

1.5.3 Mastocitosis

Las mastocitosis son un grupo de enfermedades caracterizada por una proliferación anormal de los mastocitos y la acumulación de estos en uno o más tejidos [200, 201]. Generalmente la piel es la región afectada más frecuentemente, aunque los mastocitos suelen aumentar en otros tejidos como la médula ósea, el hueso y el tracto digestivo. Las mastocitosis pueden desarrollarse en niños y adultos. Mientras que en niños normalmente se diagnostica una forma de mastocitosis cutánea (CM) que involucra solo la piel y desaparece después de la pubertad, la forma más común en adultos es la mastocitosis sistémica (SM) en la que se encuentran los mastocitos también en la medula ósea [83, 136].

Debido al origen de los mastocitos como células precursoras de la medula ósea, la World Health Organisation (WHO) clasifica las mastocitosis como una enfermedad clonar hematopoyética dentro de las enfermedades de la sangre [200]. Todos los mastocitos en la SM proceden de una sola célula neoplásica. La procedencía monoclonal se ha demostrado con la existencia de una mutación puntual en el proto-oncogén que codifica el c-kit. En la mayoría de los pacientes con SM se encuentra un cambio de los amino ácidos Asp -> Val en la posición 816 (D816V) del c-kit en las células de la medula ósea y normalmente también en las de la piel, si esta está afectada. En unos pocos pacientes las células blancas de la sangre que circulan por la periferia llevan también la mutación D816V. En cambio, no se detecta esta mutación en órganos de pacientes con CM de los cuales su origen todavía no es bien conocido [71, 110, 190, 196].

Las mastocitosis son un grupo heterogéneo de enfermedades que se distinguen por sus síntomas, la cantidad de mastocitos, el número de órganos afectados y otros aspectos. Es decir, las mastocitosis se parecen entre sí por el hecho de que la célula alterada es el mastocito, pero difieren en casi todo lo demás. La WHO ha clasificado diferentes géneros de CM y SM [200].

En los niños se distingue diferentes formas de CM, todos con buen pronóstico [83-85, 216]:

- El mastocitoma solitario, una sola lesión (a veces dos o tres) en la piel, generalmente en las extremidades, que puede producir picor [129].
- La urticaria pigmentosa, la forma más frecuente, que suele aparecer en los primeros meses de la vida o al nacer [34].
- La mastocitosis cutánea difusa, que es una forma muy poco frecuente (menos del 1% de las mastocitosis de los niños) pero la más complicada de tratar.

En los adultos se diagnostica normalmente una de las formas de SM [136, 201]:

- La SM indolente, es la forma más frecuente con afectación de la piel, la médula ósea, el tracto digestivo y los huesos. Sin embargo en todos estos tejidos el aumento de mastocitos es muy pequeño; es decir, es una enfermedad muy poco proliferativa y con buen pronóstico. Los síntomas son muy variables, el más frecuente es el picor en la piel, mientras que es más raro el enrojecimiento del pecho y la cara, el dolor abdominal o la diarrea [64, 209].

Todas las otras formas de SM son poco frecuentes y en su mayoría con mal pronóstico [198]:

- La SM asociada con otra enfermedad clonar hematológica no mastocítica
- La SM agresiva (ASM)
- La Leucemia mastocítica [54, 70]
- El Sarcoma mastocítico [94, 111]
- El Mastocitoma extracutáneo.

En las formas de las mastocitosis más frecuentes y con buen pronóstico se tratan los síntomas inducidos por los mediadores liberados de los mastocitos. Se usa medicamentos [198, 199, 213, 218]:

- ➔ que bloquean la unión de la histamina a sus receptores (*inhibidores de los receptores H1 y H2*),
- ➔ que *estabilizan la membrana del mastocito* bloqueando la liberación de los mediadores (cromoglicato de sodio) [189] o
- ➔ que *inhiben la síntesis de prostaglandinas* (ácido acetilsalicílico y los antiinflamatorios no esteroideos).

Otra forma de tratar lesiones cutáneas de las mastocitosis y el control del picor es con *radiaciones ultravioletas junto con la administración de oxoralenos* [40, 78, 105, 208].

En los casos más graves de las mastocitosis como la ASM o la leucemia se emplea una medicación antiproliferativa o citoreductiva como por ejemplo Interferon-α, Cladribina, Citosinarabinosida o Hydroxyurea [20, 193, 219].

2. OBJETIVO

El objetivo de la presente Tesis Doctoral es caracterizar las rutas que modulan la respuesta no mediada por IgE de las células HMC-1^{560} y HMC-$1^{560,816}$. Dado que la mutación en la posición 860 cambia la conformación del receptor c-Kit, se determinan las diferencias en las señales intracelulares implicadas en la activación en las dos sublíneas, en particular las interrelaciones de la señal de Ca^{2+}, pH_i y PKC. El Ca^{2+} por ser una señal bien establecida en la activación de los mastocitos, el pH por ser un activador de la respuesta que actúa de forma independiente del Ca^{2+}, y la PKC por estar funcionalmente acoplada a los niveles de Ca^{2+} y de pH.

3. PUBLICACIONES

En este capitulo se describe la parte experimental y se presentan los resultados a esta Tesis Doctoral. Está formada por un total de cinco publicaciones en las que se reflejan tanto el método experimental diseñado para abordar los objetivos propuestos como los hallazgos logrados durante la elaboración de este trabajo. La secuencia de las publicaciones es cronológica y coincide con el orden en que se han acometido los objetivos generales del trabajo.

SECCIÓN I

De la línea celular de mastocitos humanos HMC-1 existen dos sublíneas que se diferencian en mutaciones en la receptor TyrK c-kit. En esta sección se estudia la modulación de las TyrK sobre la viabilidad celular (con el test MTT), la liberación de histamina (con el metódo de Shore), el Ca^{2+} citosólico (con FURA-2) y el pH intracelular (con BCECF). Se utilizan los inhibidores de TyrK STI571, lavendustina A y genisteina en las dos sublíneas HMC-1 observandose un comportamiento parecido.
A este estudio corresponden las publicaciones:

I.A:

Influence of the Tyrosine Kinase Inhibitors STI571 (Glivec®), Lavendustin A and Genistein on Human Mast Cell Line (HMC-1^{560}) Activation

I.B:

STI571 (Glivec®) Affects Histamine Release and Intracellular pH After Alkalinisation in HMC-1$^{560, 816}$

SECCIÓN II

Se ha observado que el pH intracelular tiene una gran importancia en las células HMC-1. En esta sección se incuba las células en medios sin HCO_3^- o sin Na^+/HCO_3^-. De esta forma se definen los intercambiadores implicados en la regulación del pH_i basal. Además se determina como estos transportadores afectan a la viabilidad celular y a la liberación de histamina inducida por una alcalinización. Se observa que las dos sublíneas se comportan de modo diferente.
A este estudio corresponden las publicaciones:

II.A:

Role of Extracellular HCO_3^- in Cytosolic pH Regulation and Cell Viability of HMC-1 Human Mast Cells

II.B:

NH_4Cl Induced Alkalinisation and Exocytosis of HMC-1 Human Mast Cells are Regulated by NHE1 and a Na^+/HCO_3^- Exchanger

SECCIÓN III

Se ha observado que la PKC modula la desgranulación de mastocitos. En esta sección se estudian los efectos de la estimulación de PKC sobre la liberación de histamina, los niveles de Ca^{2+} citosólico y la expressión de c-kit (citómetro de flujo). Se compara las respuestas celulares entre las dos sublíneas HMC-1 y se observa que se comportan de forma diferente.
A este estudio corresponde la publicación:

III.A:

PKC stimulation has different effects in human mast cells HMC-1^{560} and HMC-$1^{560,816}$

3.1 SECCIÓN I

I.A: Influence of the Tyrosine Kinase Inhibitors STI571 (Glivec®), Lavendustin A and Genistein on Human Mast Cell Line (HMC-1^{560}) Activation

Journal of Cellular Biochemistry 103:1076–1088 (2008)

Influence of the Tyrosine Kinase Inhibitors STI571 (Glivec®), Lavendustin A and Genistein on Human Mast Cell Line (HMC-1^{560}) Activation

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Abstract The human mast cell line (HMC-1^{560}) was used to study the effects of tyrosine kinase (TyrK) inhibition on histamine release in consequence of intracellular Ca^{2+} or pH changes. This is important since the TyrK inhibitor STI571 (Glivec®) inhibits proliferation and induces apoptosis in HMC-1^{560}. HMC-1^{560} cells have a mutation in c-kit, which leads to a permanent phosphorylation of the KIT protein and their ligand-independent proliferation. The TyrK inhibitors STI571, lavendustin A and genistein decrease spontaneous histamine release in 24-h pre-incubated cells. Results are compared with those of the mast cell stabiliser cromoglycic acid, which also drops spontaneous histamine release. When exocytosis is stimulated by alkalinisation, STI571 pre-incubated cells release more histamine than non-pre-incubated cells. Alkalinisation-induced histamine release reaches still higher levels in STI571 cells with activated protein kinase C (PKC) by PMA. We do not observe modifications on histamine release in cells, treated with PKC inhibitors (rottlerin, Gf109203 or Gö6976). Lavendustin A- and genistein 24-h incubated cells behave similar to STI571 cells, whereas cromoglycic acid does not show effects after stimulation with alkalinisation. Stimulation of exocytosis with the Ca^{2+} ionophore ionomycin does not modify histamine response in TyrK inhibited cells. Ca^{2+} and pH changes are observed after long-time incubation with STI571. Results show that pH is still higher in STI571 pre-incubated cells after alkalinisation with NH_4Cl, whereas intracellular Ca^{2+} concentration remains stable. This work further strength the importance of pH_i as a cell signal and suggest that STI571 has transduction pathways in common with other TyrKs. J. Cell. Biochem. 103:1076–1088, 2008.

Key words: HMC-1; mast cells; STI571; Glivec; lavendustin; genistein; alkalinisation; intracellular pH; calcium; tyrosine kinase; protein kinase C

Mast cells are a cell type often used in signal transduction studies because they secrete their granules through a fast exocytotic process, which can be used as a functional model. Up to now, only two human mast cell lines are available: the LAD 1/2 line (Laboratory of Allergic Diseases) [Kirshenbaum et al., 2003] and the human mast cell line (HMC-1) [Butterfield et al., 1988]. HMC-1 presents a very regular growth and behaviour and there are still many unknown aspects concerning to its activation process.

HMC-1 cells express in their membrane the tyrosine kinase (TyrK) receptor KIT. KIT is a member of the type III transmembrane Tyr kinases with an extracellular domain that binds to mast cell growth factor, also known as steel factor and stem cell factor (SCF) [Linnekin, 1999; Longley et al., 1999; Heinrich et al., 2000]. The extracellular region consists of five immunoglobulin-likes binding repeats, three of them are involved in SCF-binding. Ligand binding results in dimerisation and phosphorylation of KIT and leads to an activation of its intrinsic intracellular TyrK activity. A juxtamembrane domain and two TyrK domains, which are separated by a kinase insert, are situated in the intracellular part of KIT [Linnekin, 1999]. Signals mediated by the extracellular

Abbreviations used: HMC-1, human mast cell line; TyrK, tyrosine kinase; SCF, stem cell factor; Ca^{2+}, calcium; NH_4Cl, ammonium chloride; PKC, protein kinase C.

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receptor influence important processes like proliferation, differentiation, migration, activation and survival of mast cells [Sundstrom et al., 2003]. Mutations in the proto-oncogene c-kit, which encodes the KIT protein, evoke a ligand independent proliferation of HMC-1 [Buchdunger et al., 2000]. HMC-1^{560} has one mutation that results in a Gly560 → Val amino acid change. HMC-$1^{560,816}$ has the 560 mutation and one more in Asp816 → Val. These mutations lead to a constitutive activation of the KIT protein that has been found in several types of human malignant diseases [Ma et al., 2002].

The 560 mutation could be detected in cells of patients with gastrointestinal stromal tumour (GIST), cutaneous mastocytosis (CM) and in a few cases of systemic mastocytosis (SM). Mastocytosis is a mast cell disease characterised by an abnormal growth and accumulation of mast cells in one or more tissues. CM is common in children. Typical clinical and histological skin lesions and the absence of definitive signs of systematic involvement define it [Valent et al., 2001]. Cells with 816 mutations have been found in more than 80% of patients with SM. The grave disease appears commonly in adults and its diagnosis is based on multifocal histological lesions in the bone marrow or other extra-cutaneous organs. The detection of the activating c-kit point mutation at codon 816 is one diagnostic criterion of SM [Valent et al., 2001; Garcia-Montero et al., 2006]. Different mutations of KIT have a high relevance in the pathology and identification of distinct forms of SM and their modulation become major significance for its treatment and prognosis [Valent et al., 2001].

The TyrK inhibitor STI571, also known as Glivec® and imatinib, was discovered during the testing of compounds for inhibition of protein kinase C (PKC) [Buchdunger et al., 1996]. The development of a number of chemical analogs resulted in STI571, shown to be an inhibitor of platelet-derived growth factor receptor, inhibitor of v-Abl, c-Abl, Bcr-Abl and c-kit protein-TyrKs [Buchdunger et al., 2000]. STI571 acts as a competitive inhibitor of adenosine triphosphate (ATP). It binds to a portion of the ATP-binding site of the kinase in its inactivated conformation and keeps it in this frozen-like condition [Shah et al., 2006]. STI571 is successful used in mast cell diseases involving the wild-type c-kit by inhibition of the SCF-dependent kinase activation. The inhibitory effect is still higher in mutants with Gly560Val substitution, by decreasing the autophosphorylation of the mutant KIT through inhibition of the kinase activity rather than by down-regulating expression of c-kit protein. Therefore, it is successfully used in patients with GIST [Heinrich et al., 2000]. Unfortunately, it is ineffective in cells with Asp816Val mutation that appear in the majority of aggressive SM and mast cell leukaemia [Ma et al., 2002].

The aim of this work was to study the relationship between HMC-1^{560} activation and the TyrK pathway, especially that of KIT. We used STI571 and the non-specific TyrK inhibitors, lavendustin A and genistein to investigate how they affect histamine release in consequence of changes in intracellular Ca^{2-} or pH in these cells. The availability of two cultured cell lines that differ in one mutation is an opportunity for further investigation of the influence of this mutation on signalling pathways. A better understanding could lead to more efficient drug targeting. We used HMC-1^{560} cells that appear in few patients with SM. Similar studies should be made with HMC-$1^{560,816}$ cells that can be found in mast cell neoplasm, to clarify the effect of the 816 mutation on appointed pathways.

METHODS

Chemicals

Ammonium chloride (NH_4Cl) was from Panreac (Barcelona, Spain); rottlerin, ionomycin, Gö6976, GF109203X, genistein and lavendustin A were from Alexis Corporation (Läufelfingen, Switzerland); 2,7-bis (carboxyethyl)-5(6)carboxyfluorescein-acetoxymethylester (BCECF AM) and FURA-2 AM were from Molecular Probes (Leiden, The Netherlands). Phorbol 12-myristate 13-acetate (PMA), Cromoglycic acid and MTT (3-[4,5-dimethyldiazol-2-yl]-2,5-diphenyltetrazolium bromide) were from Sigma-Aldrich (Madrid, Spain). STI571 was provided by Dr. Luis Escribano Mora (Servicio de Hematología, Hospital Ramón y Cajal, Madrid).

Cell Cultures

HMC-1 cells were kindly provided by Dr. J. Butterfield (Mayo Clinic, Rochester, MN) and maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% foetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells were

expanded weekly and not more than 40 passages.

Cell Preparation

For histamine release assays, cells were centrifuged (1,500 r.p.m., 5 min, 4°C) and washed twice with saline solution (1,000 r.p.m., 5 min, 4°C). The composition of this solution was (mM): Na^- 142.3; K^- 5.94; Ca^{2-} 1; Mg^{2-} 1.2; Cl^- 126.2; HCO_3^- 22.85; HPO_4^{2-} 1.2, SO_4^{2-} 1.2; glucose 1 g/L.

For Ca^{2-} and pH measurements cells were treated in the same conditions, but washed in saline solution plus 0.1% bovine serum albumin (BSA).

The incubation medium was equilibrated with CO_2 prior to use. During the experiments, pH (7.20) was maintained constant by bubbling CO_2. Experiments were carried out at least three times, by duplicate, both for histamine release assays and Ca^{2-} and pH measurements.

Cell Incubation

Freshly prepared concentrated solution (6.2 μl) of each drug (40 times concentrated) were added to the incubation medium to attain a final volume of 150 μl and pre-incubated. When the medium reached 37°C, 100 μl of a cell suspension with an approximate density of $1.5–2 \times 10^6$ cells/ml were added to each tube. Incubations were carried out in a bath at 37°C for 10 min.

The incubations were stopped by immersing the tubes in a cold bath. After centrifugation at 2,300 r.p.m. for 10 min, the supernatants were collected and decanted into other tubes for histamine determination. Appropriate controls to determine spontaneous histamine release in the absence of stimuli were executed in every experiment.

Histamine Release Assays

Histamine release was tested with a spectrofluorometer, (Fluoroskan II, Labsystems, Barcelona, Spain) in both pellet (non-released histamine) and supernatant (released histamine) according to Shore's method [Shore, 1971]. To form the fluorescent complex 0.04% orthophthaldialdehyde was used, also trichloroacetic acid (14%) to avoid protein interferences in the histamine release determination. To ensure total histamine, pellets were sonicated for 60 s in 0.2 ml of 0.1 N HCl. In NH_4Cl experiments, histamine release was measured only in pellets, since this compound interferes with the fluorescent complex. Results shown were expressed as the percentage of released histamine from the total histamine content.

Cell Viability

After exposure to different concentrations of STI571 during 24 h in culture medium, cells were centrifuged (1,500 r.p.m., 5 min, 4°C). The pellets were resuspended in saline solution with MTT (250 μg/ml) and incubated at 37°C for 30 min in darkness.

After washing twice with saline solution cells were sonicated in water for 60 s. The coloured formazan salt was measured at 595 nm in a spectrophotometer plate reader.

Measurement of Cytosolic Free Ca^{2-} and Intracellular pH

HMC-1 cells were loaded with FURA-2 AM (0.2 μM) and BCECF AM (0.05 μM) in a bath at 37°C, for 10 min. After this time, loaded cells were washed with saline solution (1,000 r.p.m., 10 min, 4°C). Cells were attached to glass coverslips treated with poly-L-lysine, and these were inserted into a thermostated chamber (Life Sciences Resources, UK). Cells were viewed using a Nikon Diaphot 200 microscope equipped with epifluorescence optics (Nikon 40x—immersion UV—Fluor objective). Addition of drugs was made by aspiration and addition of fresh bathing solution to the chamber. Intracellular pH and cytosolic Ca^{2-} concentration were obtained from the images collected by fluorescence equipment (Life Sciences Resources). The light source was a 175 W xenon lamp, and the used wavelengths were selected with filters. For FURA-2 AM, the excitation wavelengths were 340 and 380 nm, with emission at 505 nm; for BCECF AM the excitation was performed at 440 and 490 nm, with 530 nm for emission. The calibration of the fluorescence values versus intracellular Ca^{2-} was made according to the method of Thomas et al. [1979] and of fluorescence values versus pH as per Grynkiewicz et al. [1985]. In brief, a calibration curve was obtained with four known values of pH, measuring the fluorescence ratio obtained in the presence of nigericin, in a K^- solution, for each pH value.

Statistical Analysis

Results were analysed using the Student's *t*-test for unpaired data. A probability level of

0.05 or smaller was used for statistical significance. Results were expressed as the mean ± SEM.

RESULTS

Following to our previous work in HMC-1^{560} cells [Pernas-Sueiras et al., 2005, 2006a,b], we checked the effect of TyrK inhibitors on histamine release induced by alkalinisation and ionomycin.

After 10 min of incubation with one of the TyrK inhibitors STI571 (10 μM), lavendustin A (1 μM) and genistein (10 μM), we stimulated the cells with different concentrations of NH_4Cl or the Ca^{2-} ionophore ionomycin [Pernas-Sueiras et al., 2005]. Neither alkalinisation (Fig. 1A–C)

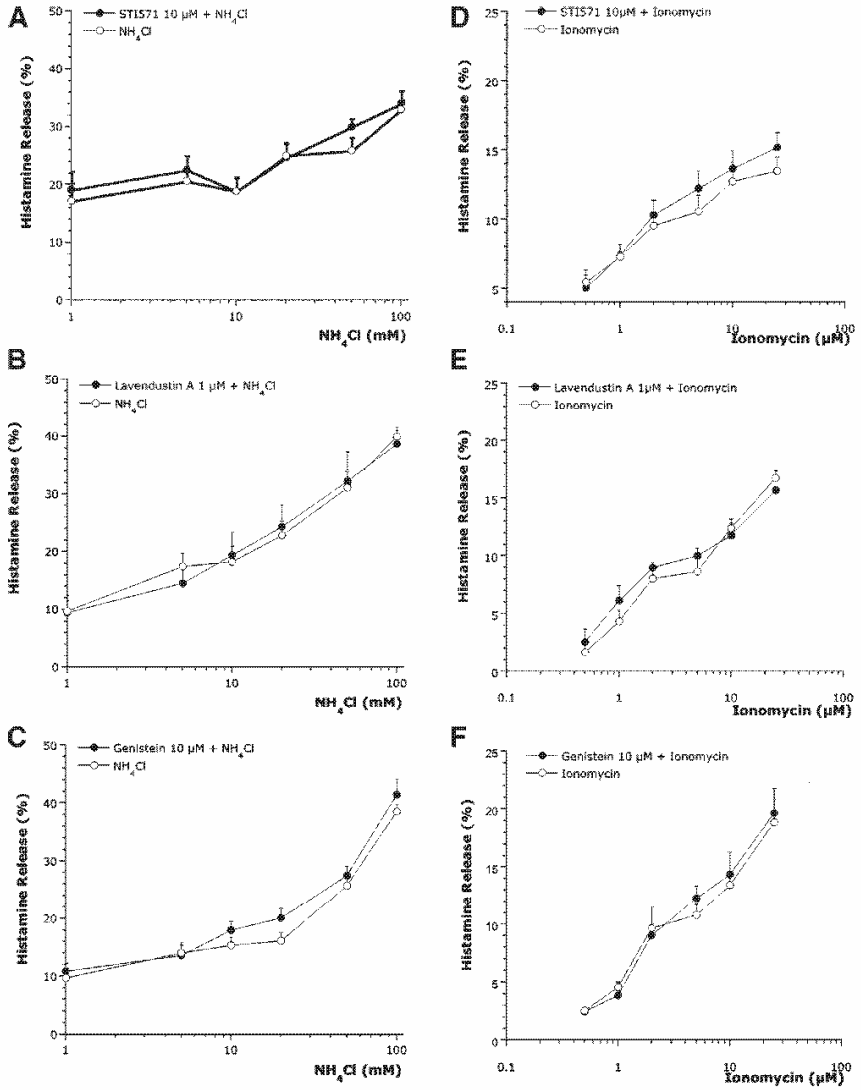

Fig. 1. Effect of TyrK inhibition on histamine release. HMC-1^{560} cells were pre-treated during 10 min in saline solution with STI571 (10 μM; **A**,**D**), lavendustin A (1 μM; **B**,**E**), genistein (10 μM; **C**,**F**) and afterwards stimulated with different concentrations of NH_4Cl (A–C) o ionomycin (D–F). Mean ± SEM of four experiments.

nor stimulation with ionomycin (Fig. 1D–F) induced any change in histamine release.

Next, we investigated the time dependence of TyrK inhibition in HMC-1^{560}. We tested STI571 viability by MTT assays in cells 24 h incubated with different concentrations of the drug. Our results confirmed those of other studies that HMC-1^{560} cell died with increased drug presence [Heinrich et al., 2000; Akin et al., 2003] (Fig. 2). From this graphic, we selected the concentration 25 nM where 70% of the cells survived but STI571 still had an effect.

We incubated HMC-1^{560} cells 24 h with this concentration and a control without drug in culture medium. Afterwards histamine assays were realised as described above. Our results in Figure 3A show that spontaneous histamine release was significantly decreased in STI571 (25 nM) 24 h pre-incubated cells. We obtained similar results with cells pre-treated with lavendustin A (1 μM; Fig. 3B) and genistein (1 μM; Fig. 3C), two unspecific TyrK inhibitors. Hereon, histamine response of cromoglycic acid (100 μg/ml) pre-incubated cells was checked. Its salt sodium cromoglycate prevents mast cell degranulation [Theoharides et al., 1980; Edwards and Howell, 2000]. When this drug was present during 24 h spontaneous histamine release was also decreased (Fig. 3D).

Furthermore, histamine release assays were made with NH_4Cl (50 mM) to compare the TyrK inhibited cells with control cells, since alkalinisation is known to stimulate histamine release in this cell line [Pernas-Sueiras et al., 2005]. As Figure 4A shows, it is surprising that STI571 pre-incubated cells released more histamine after alkalinisation. HMC-1^{560} cells pre-treated with lavendustin A (1 μM; Fig. 4B) and genistein (1 μM; Fig. 4C) showed similar behaviour, whereas cromoglycic acid did not affect histamine release induced by alkalinisation (Fig. 4D).

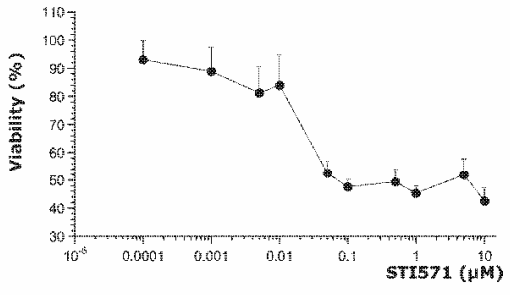

Fig. 2. HMC-1^{560} viability after 24 h incubation with STI571. Different concentrations of STI571 were added in culture medium and cells were incubated for 24 h at 37°C. Cell viability was checked by MTT test. Control cells with non-STI571 treatment, were used as 100% viability and ethanol treatment as 100% dead, in each experiment. Mean ± SEM of four experiments.

The Ca^{2-} ionophore ionomycin is another stimulus that induces histamine release in HMC-1^{560} [Pernas-Sueiras et al., 2005]. STI571 pre-incubation on further stimulation with ionomycin (2 μM), Figure 4E, did not change histamine response compared to ionomycin treatment only. In the same way, the other TyrK inhibitors lavendustin A (Fig. 4F) and genistein (Fig. 4G) did not show differences when comparing pre-treated cells with control cells after ionomycin stimulation. In addition, cromoglycic acid, Figure 4H, did not affect histamine release induced by the Ca^{2-} ionophore.

To check if the increased histamine release was a consequence of intracellular Ca^{2-} or pH changes, we observed both parameters in TyrK inhibited cells. HMC-1^{560} cells were pre-incubated with STI571 (25 nM) during 24 h like in histamine release assay experiments. We performed the experiments in Ca^{2-}-free medium and restored the ion later on. After obtaining a baseline, we added NH_4Cl (50 mM). As Figure 5A shows, cytosolic pH increased at the addition point of the compound and slowly decreased afterwards. After adding Ca^{2-} (1 mM) to the medium, we could not observe any other pH_i changes. As the same figure shows, pH increase was higher in STI571 pre-incubated cells than in not treated cells. The difference was statistically significant. The intracellular Ca^{2-} concentration remained stable during the whole experiment (Fig. 5B).

The same experiment was performed by adding ionomycin (0.1 μM) instead of NH_4Cl. As expected, the cytosolic Ca^{2-} increased at the ionomycin addition point because intracellular reservoirs were depleted (Fig. 5D). Thereupon the Ca^{2-} concentration steadily decreased until Ca^{2-} (1 mM) was added to the external medium. Cytosolic Ca^{2-} rapidly rose through the fast influx of the cation. As Figure 5C shows, the intracellular pH remained stable until Ca^{2-} addition. After that we could observe a lightly, not significantly alkalinisation in ionomycin stimulated cells. However, no significant differences between STI571 treated and control cells were seen neither in intracellular Ca^{2-} nor in

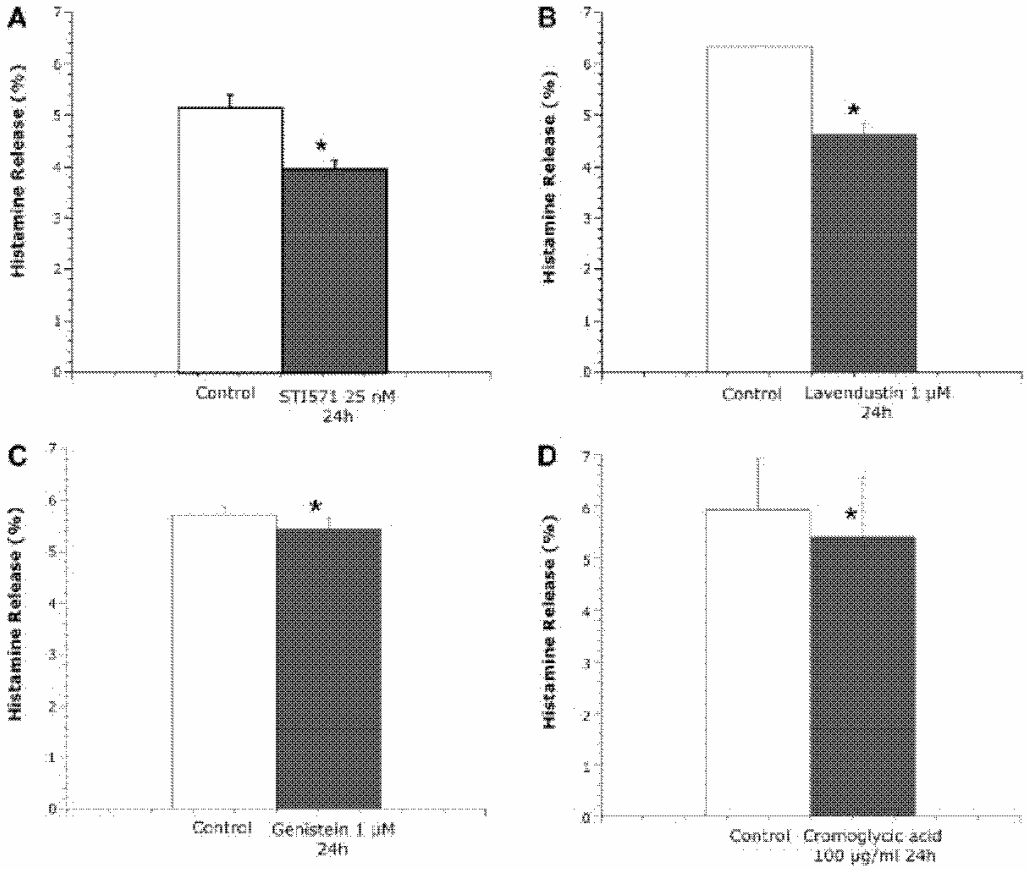

Fig. 3. Effect of TyrK inhibitors and cromoglycic acid on HMC-1^{560} spontaneous histamine release after 24 h of incubation. Cells were pre-incubated for 24 h in the presence of STI571 (25 nM; **A**), lavendustin A (1 µM; **B**), genistein (1 µM; **C**) or cromoglycic acid (100 µg/ml; **D**) in culture medium. Histamine release was checked after 10 min of incubation in saline solution (37°C). Mean ± SEM of four experiments. * Significant differences between control and drug pre-incubated cells.

pH changes after ionomycin stimulation (Fig. 5C,D).

From previous studies, we know that PKC stimulation by the phorbol ester PMA induces release of histamine after NH_4Cl stimulation [Pernas-Sueiras et al., 2006a]. As Figure 6A shows, STI571 significantly increased alkalinisation-induced histamine release after treatment with PMA (100 ng/ml). Pre-treatment of HMC-1^{560} with lavendustin A (Fig. 6B) or genistein (Fig. 6C) had the same effect. Cromoglycic acid (Fig. 6D) did not affect histamine response.

In contrast to alkalinisation, STI571 did not change the histamine release in ionomycin-stimulated cells in combination with PMA (Fig. 6E). Neither lavendustin A, Figure 6F, nor genistein, Figure 6G, had effects. Figure 6H shows that the mast cell stabiliser cromoglycic acid did not affect histamine release.

Accordingly, we checked intracellular Ca^{2-} and pH profiles when PKC was stimulated. As Figure 7A shows, pH increased after NH_4Cl (50 mM) addition to a Ca^{2-}-free medium. When Ca^{2-} (1 mM) was restored to the medium, no effect was observed on the descending pH. We could see a slightly increased pH in STI571 pre-incubated cells, although that was not statistically significant. During the whole experiment, in both, STI571 pre-incubated and control cells, Ca^{2-} levels remained stable (data not shown).

In those experiments of ionomycin in combination with PMA, no changes were observed in

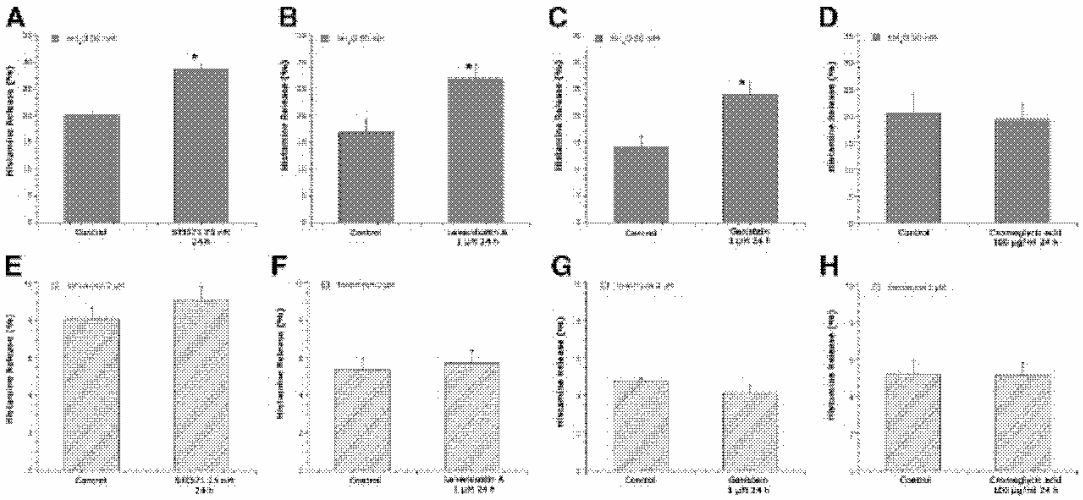

Fig. 4. Effect of TyrK inhibitors and cromoglycic acid on alkalinisation- and ionomycin-induced histamine release in HMC-1^{560} cells. Cells pre-incubated with STI571 (25 nM; **A**), lavendustin A (1 μM; **B**), genistein (1 μM; **C**) or cromoglycic acid (100 μg/ml; **D**) during 24 h were stimulated with NH_4Cl (50 mM) during 10 min in saline solution (37°C). Cells pre-incubated with STI571 (25 nM; **E**), lavendustin A (1 μM; **F**), genistein (1 μM; **G**) or cromoglycic acid (100 μg/ml; **H**) during 24 h were stimulated with ionomycin (2 μM) during 10 min in saline solution (37°C). Mean ± SEM of four experiments. * Significant differences between control and drug pre-incubated cells.

pH_i at the point where drug was added (data not shown). Results of cytosolic Ca^{2-} changes in Figure 7B show an expected increase after adding ionomycin (0.1 μM) and a subsequent drop. The Ca^{2-} (1 mM) restoration to the external medium induced another but higher increase in intracellular Ca^{2-} concentration due to the extracellular entry of the cation.

Furthermore to proof the influence of PKC inhibition we performed histamine assays in STI571 (25 nM) 24 h pre-incubated cells using the PKC inhibitors Gö6976 (100 nM), GF109203X (500 nM) and rottlerin (10 μM). We could not find any significant alteration between TyrK blocked and control cells, neither after alkalinisation (Fig. 8A–C) nor after stimulation with ionomycin (Fig. 8D–F).

DISCUSSION

In previous works in HMC-1^{560}, we studied transduction pathways that have influence in intracellular Ca^{2-} and pH as activation signals for release of histamine. The object of this study was to characterise signalling pathways mediated by TyrKs. This is important since the KIT TyrK inhibitor STI571 is able to induce apoptosis and to inhibit proliferation in cells with Gly560 → Val amino acid change in KIT. We wanted to clarify especially the influence of TyrK-activity on exocytotic process.

In HMC-1^{560}, KIT is permanently phosphorylated and SCF independently activated. It is involved in a variety of intracellular signalling pathways like that of phosphatidylinositol-3′-kinase (PI3K) and mitogen activated protein kinases (MAPK) [Sundstrom et al., 2003]. Furthermore, it is suggested that JAK/STAT and Scr play an important role in the proliferation process induced by KIT in HMC-1 like it has been shown in other cell lines [Linnekin, 1999]. To modulate these processes we inhibited the catalytic centre of the intracellular KIT TyrK with STI571, which acts as a competitive inhibitor of ATP [Shah et al., 2006]. Subsequently connected pathways as those mentioned above are blocked. The efficacy of the drug shows its use in GIST [Ma et al., 2002] and the viability test in this work and other studies [Heinrich et al., 2000]. HMC-1^{560} cells died with increased drug presence.

All these studies were long-term studies. When we incubated the cells for 10 min with STI571 or the two unspecific TyrK inhibitors lavendustin A and genistein, we could not observe any change in histamine release.

From the viability test, we selected the concentration 25 nM of STI571 that allowed

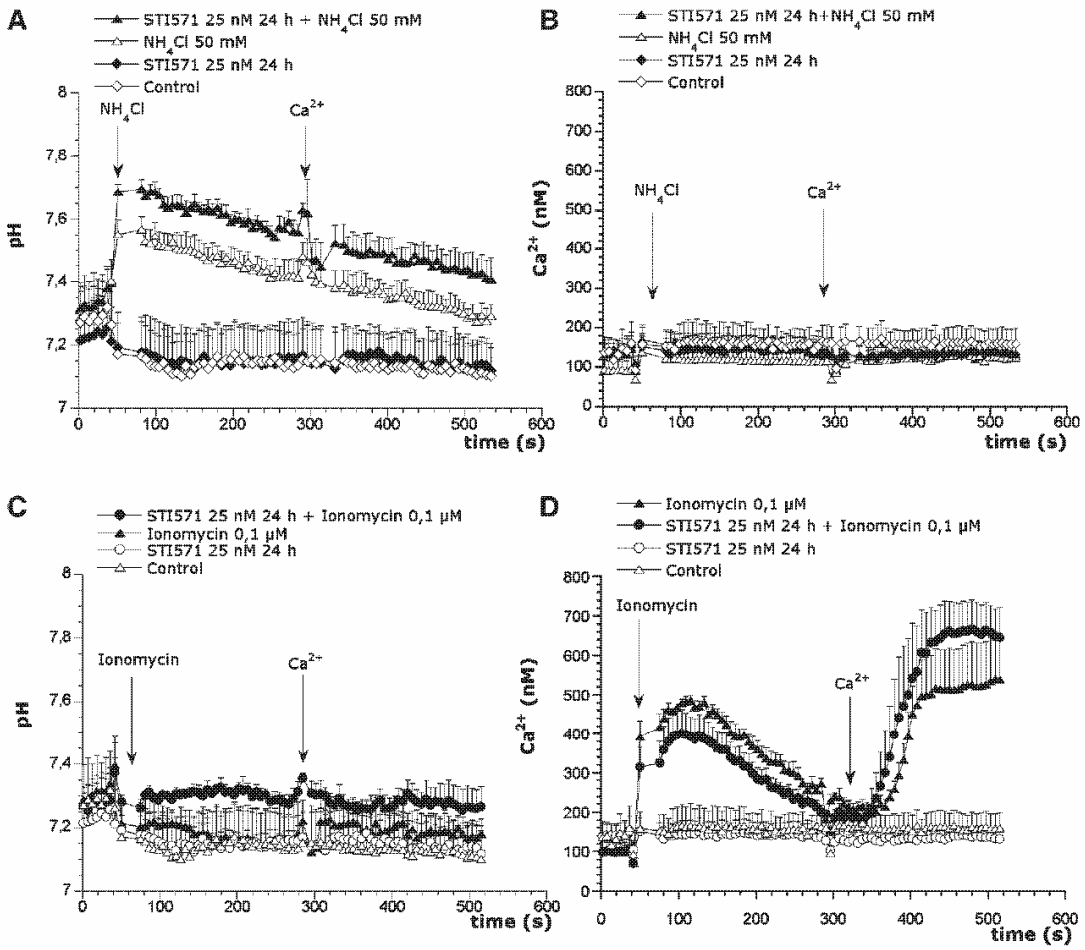

Fig. 5. Effect of STI571 on intracellular Ca^{2+} and pH levels in HMC-1^{560} cells. Cells were pre-incubated for 24 h in presence of STI571 (25 nM) in culture medium. Cytosolic pH and Ca^{2+} changes were observed after NH_4Cl (50 mM; **A**,**B**) and ionomycin (0.1 µM) addition (**C**,**D**). The first arrow indicates drug addition and second that extracellular Ca^{2+} (1 mM) was restored. Mean ± SEM of four experiments.

70% of the cells to survive but still showed an effect. We expected that histamine release would decrease after 24-h pre-incubation with STI571. Our results confirmed a lower spontaneous histamine response. We made similar experiments with lavendustin A and genistein, since these drugs are reported to diminish histamine release in human basophiles [Tedeschi et al., 2000]. Lavendustin A and genistein affected spontaneous histamine release of HMC-1^{560} cells like STI571. We suggested, this might be due to the block of above-mentioned TyrK connected transduction pathways as PI3K and JAK/STAT.

To rule out the possible role of the TyrK inhibitors as membrane stabilisers, we treated the cells for 24 h with cromoglycic acid, a drug often used in treatment of diseases with high transmitter release such as different kind of allergies, asthma and mastocytosis [Shin et al., 2004]. Its mechanism of action was thought to be the stabilisation of the mast cell membrane and subsequent the prevention of transmitter release [Theoharides et al., 1980; Edwards and Howell, 2000]. Further on the drug was shown to inhibit the activation of human neutrophils, eosinophils and monocytes in vitro and to reduce IgE production [Kimata et al., 1991;

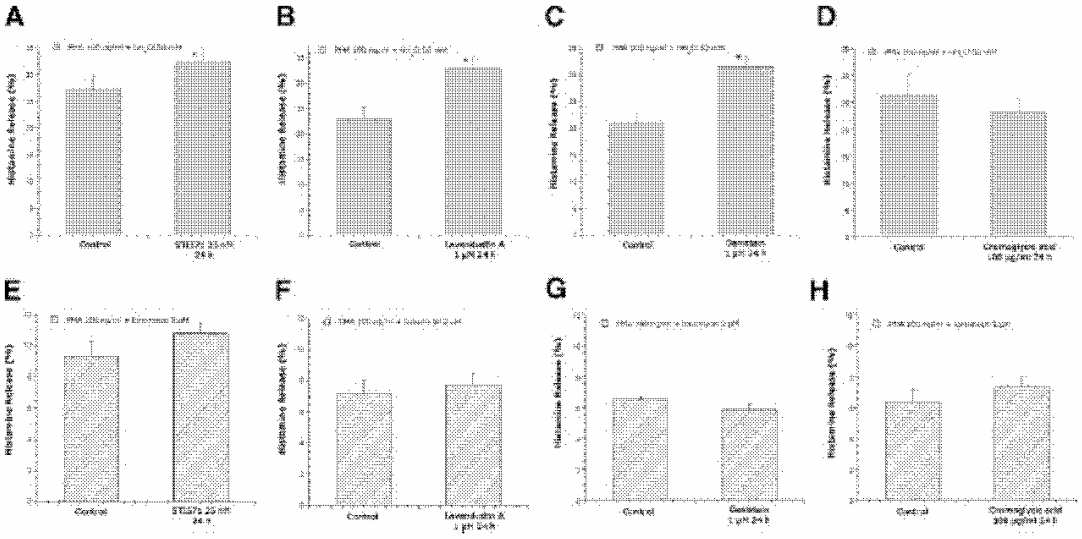

Fig. 6. Effect of PKC activation and TyrK inhibition o cromoglycic acid incubation on alkalinisation and ionomycin induced histamine release in HMC-1^{560}. Cells pre-incubated with STI571 (25 nM; **A**), lavendustin A (1 μM; **B**), genistein (1 μM; **C**) or cromoglycic acid (100 μg/ml; **D**) during 24 h were pre-treated with PMA (100 ng/ml) 10 min and afterwards stimulated with NH_4Cl (50 mM) during 10 min in saline solution (37°C). Cells pre-incubated with STI571 (25 nM; **E**), lavendustin A (1 μM; **F**), genistein (1 μM; **G**) or cromoglycic acid (100 μg/ml; **H**) during 24 h were pre-treated with PMA (100 ng/ml) for 10 min and afterwards stimulated with ionomycin (2 μM) during 10 min in saline solution (37°C). Mean ± SEM of four experiments. * Significant differences between control and drug pre-incubated cells.

Loh et al., 1994]. An interesting approach was that cromolyn might inhibit histamine secretion by phosphorylation of a mast cell protein, which is involved in exocytosis regulation. PKC inhibitors and a cation ionophore blocked this phosphorylation [Correia et al., 1996; Wang et al., 1999]. We found as well less histamine release in unstimulated cells treated with cromoglycic acid. However, results of experiments with cromoglycic acid differed of those of the TyrK-inhibitors after stimulation with NH_4Cl or ionomycin. Therefore, we discarded our hypothesis that STI571 could act in a cromolyn like manner.

In HMC-1^{560}, histamine release increases after stimulation with NH_4Cl simultaneously with an increase of intracellular pH, and at the same time Ca^{2-} levels remain stable [Pernas-Sueiras et al., 2005]. In rat peritoneal mast cells [Wan et al., 2005] and human basophiles [Tedeschi et al., 2000], TyrK inhibition leads to a decrease of histamine release after stimulation. Surprisingly STI571 pre-treated HMC-1^{560} cells released more histamine after alkalinisation than control cells. As far as we know, this is the first time that describes that TyrK inhibition increased histamine release in human mast cells.

We could not explain the stimulation of exocytosis after alkalinisation with the KIT kinase inhibition, because as previous mentioned, inhibition should occur downstream the activation process. Therefore, we performed experiments with the unspecific TyrK inhibitors, lavendustin A and genistein in the same fashion as with STI571. Especially genistein has been tested earlier to decrease histamine release after stimulation in different mast cell models [Tedeschi et al., 2000; Wan et al., 2005]. However, alkalinisation induced histamine release rose after 24-h treatment with both drugs. This suggests that STI571 has transduction pathways in common with other Tyr kinases, at least as evidenced with lavendustin A and genistein in this cell line.

To investigate this behaviour, intracellular Ca^{2-} and pH levels were observed in STI571 pre-incubated and control cells. Our results showed that in pre-treated cells pH_i significantly increased more after alkalinisation. This matches with our previous statements that alkalinisation induces histamine release in this

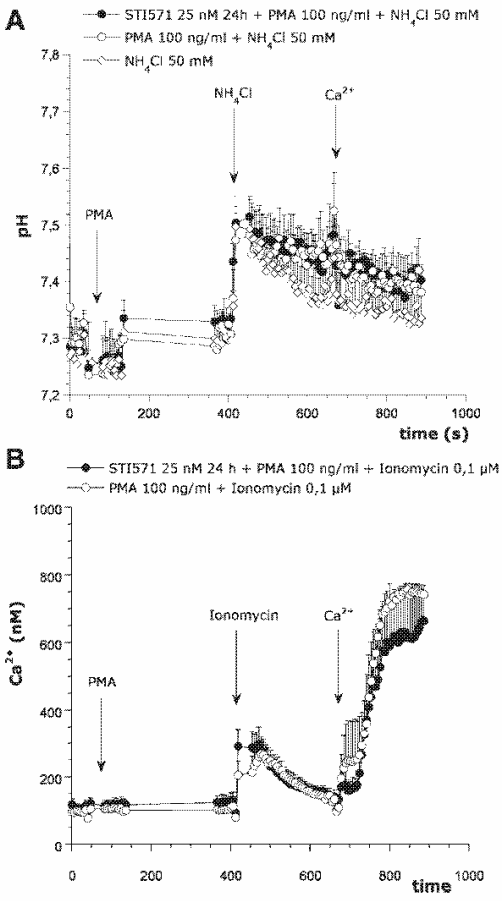

Fig. 7. Effect of PKC activation and STI571 on alkalinisation-modulated intracellular pH and Ca^{2+} levels in HMC-1^{560} cells. Cells were pre-incubated for 24 h in the presence of STI571 (25 nM) in culture medium. NH_4Cl (50 mM) was added and cytosolic pH changes were observed (**A**). Cytosolic Ca^{2+} changes were observed after ionomycin 0.1 µM addition (**B**). The first arrow indicates the addition of PMA (100 ng/ml), the second arrow indicates stimulator addition and the third that extracellular Ca^{2+} (1 mM) was restored. Mean ± SEM of three experiments.

cell line [Pernas-Sueiras et al., 2005]. Observing intracellular Ca^{2-} in the same experiments, we did not find any difference in STI571 and control cells. This suggests once more that pH is a stimulating signal to induce exocytosis in HMC-1^{560} and that STI571 influences somehow this signalling pathway. It has been described in cardiomyocytes that ATP is a strong activator of Src/Tyr kinases and of the anion Cl^-/HCO_3^- exchanger. TyrK activation results in intracellular acidosis [Puceat et al., 1998; de la Rosa et al., 2001]. Src TyrK inhibition by genistein leads to an inhibition of Cl^-/HCO_3^- exchanger and this prevents ATP-induced acidification. It is necessary to bear in mind that STI571 inhibits the ATP binding site of KIT and that KIT is connected to other Tyr kinases. It might be that TyrK inhibition increases intracellular pH by inhibiting the anion Cl^-/HCO_3^- exchanger, similarly as in cardiomyocytes, what results in increased histamine release after alkalinisation. Alkalinisation induced by TyrK inhibition could be part of the initiation of the apoptotic process that induces STI571 in these cells. Further studies should be done, including apoptosis activation experiments, to investigate the impact of alkalinisation in cellular death.

Cytosolic Ca^{2-} concentrations play a role in exocytotic processes since the Ca^{2-} ionophore ionomycin induces histamine release by modulating intracellular Ca^{2-} concentration [Pernas-Sueiras et al., 2005, 2006b]. Therefore, similar experiments using ionomycin instead of NH_4Cl were performed. Whereas histamine release was significantly elevated after alkalinisation in STI571 pre-treated cells, the increase after ionomycin stimulation was not significantly. Results obtained in the microscope with ionomycin in STI571 pre-incubated cells confirmed those of histamine release assays. There were no significant differences in Ca^{2-} and pH changes between STI571 and control cells.

These results demonstrate that STI571 influences exocytosis of HMC-1^{560} by a pH-, but not by a Ca^{2-}-dependent pathway. To underpin this statement we further studied the modulation of PKC, since PMA induced an increased histamine release in NH_4Cl and ionomycin stimulated cells [Pernas-Sueiras et al., 2006a]. Alkalinisation induced exocytosis took place without modulating cytosolic Ca^{2-} levels in cells pre-treated with PMA. We demonstrated that a long-term pre-incubation with one of the TyrK inhibitors induced a significantly higher release of histamine in PKC stimulated cells after alkalinisation. STI571 pre-incubated cells showed a slightly higher initial pH after NH_4Cl addition. Even if the difference was not significant, we suggest that this is once more a reason for the increased histamine release of STI571 pre-incubated cells. We could not find differences observing intracellular Ca^{2-} concentrations. Pre-treated cells with activated PKC, stimulated by ionomycin did not show

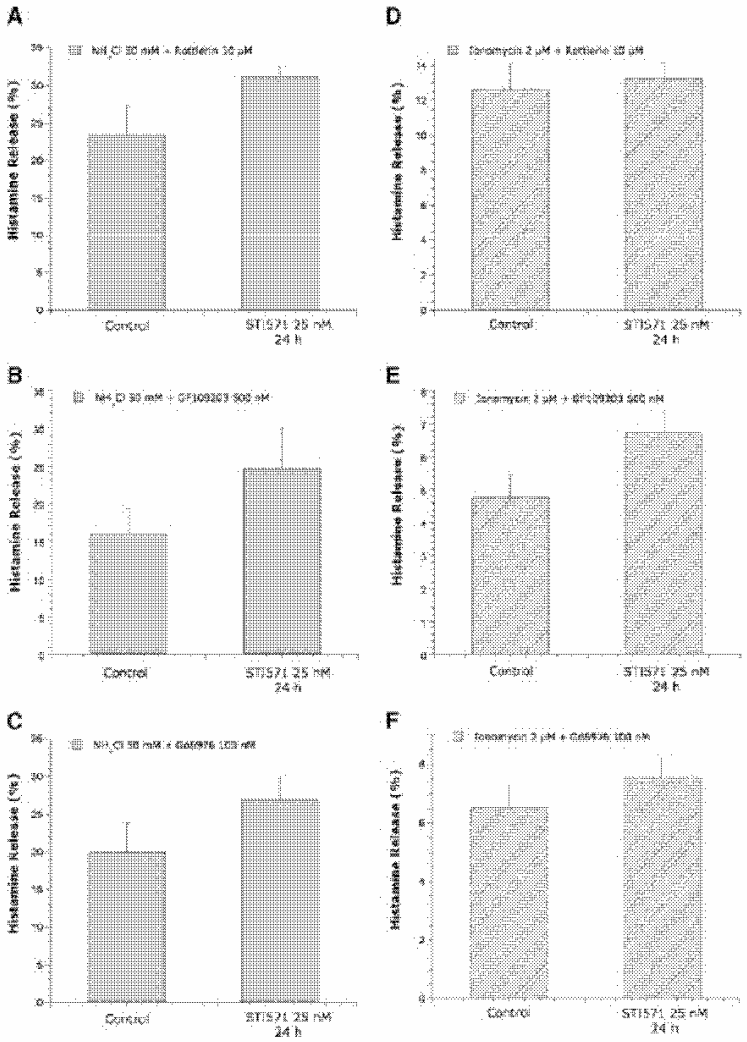

Fig. 8. Effect of PKC inhibition and STI571 on alkalinisation or ionomycin induced histamine release in HMC-1^{560}. Cells pre-incubated with STI571 (25 nM) during 24 h were pre-treated with rottlerin (10 μM), Gö6976 (100 nM), GF109203X (500 nM) for 10 min and afterwards stimulated with NH_4Cl (50 mM; **A**–**C**) or with ionomycin (2 μM; **D**–**F**) during 10 min in saline solution (37°C). Mean ± SEM of four experiments.

differences neither concerning to histamine release nor to intracellular pH and Ca^{2-} changes. Results of STI571 pre-incubated and control cells, when PKC was inhibited by rottlerin, GF109203X or Gö6976, did not differ from each other concerning to histamine release.

Nevertheless, the fact that PMA-increased alkalinisation induced histamine release in TyrK inhibited cells let us suggest that there exists a connection between PKC and TyrK pathways in HMC-1^{560}.

Our results demonstrate that the TyrK inhibitors STI571, lavendustin A and genistein inhibit in this cellular model similar pathways. They are able to influence intracellular pH levels, and this leads in the case of HMC-1^{560} to an increased histamine release after alkalinisation. Furthermore like in previous studies, we show the importance of intracellular pH in HMC-1^{560} and that can be by itself enough signal to activate exocytosis [Alfonso et al., 2000; Pernas-Sueiras et al., 2005]. Our study may have potential implications to modify the

effect on the clinical use of STI571. It should be noticed that by decreasing the amount of HMC-1^{560} cells by using STI571, surviving cells could be more reactive after their stimulation.

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I.B: STI571 (Glivec®) Affects Histamine Release and Intracellular pH After Alkalinisation in HMC-1[560,816]

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STI571 (Glivec®) Affects Histamine Release and Intracellular pH After Alkalinisation in HMC-$1^{560, 816}$

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Abstract The human mast cell line (HMC-$1^{560, 816}$) was used to study the effect of the tyrosine kinase inhibitor STI571 (Glivec®) on exocytosis, intracellular Ca^{2+} and pH changes, because STI571 inhibits the proliferation of HMC-1^{560} and induces its apoptosis. This drug does not have these effects on HMC-$1^{560, 816}$. Exocytosis in HMC-$1^{560, 816}$ cells can be stimulated by alkalinisation with NH_4Cl as well as with ionomycin. Surprisingly 24-h pre-incubation with STI571 decreases spontaneous histamine release of HMC-$1^{560, 816}$ cells, but increases the histamine response after alkalinisation and not after ionomycin-stimulation. After addition of NH_4Cl, pH_i has a higher increase in STI571 pre-incubated cells, without changing intracellular Ca^{2+} concentration. Activation of PKC in combination with tyrosine kinase inhibition increases also histamine release in HMC-$1^{560, 816}$ cells. Strangely, STI571 pre-incubated cells with PKC inhibited by rottlerin show the same effects. In these cells, cytosolic pH increases more than in control cells. This is the first report of STI571 effect in HMC-$1^{560, 816}$ cells. It seems that different pathways modulate signals for proliferation and exocytosis. STI571 does not only inhibit KIT TyrK, but may also influence cytosolic pH after alkalinisation in both cell lines, HMC-1^{560} and HMC-$1^{560, 816}$, and this ends in induced histamine release. This work is important since HMC-$1^{560, 816}$ cells are reported in 80% of aggressive systemic mastocytosis cases and the understanding of some signalling pathways involved in mast cell response could facilitate drug targeting. J. Cell. Biochem. 103: 865–876, 2008.

Key words: HMC-1^{560}; HMC-$1^{560, 816}$; mast cells; STI571; Glivec; alkalinisation; intracellular pH; calcium; tyrosine kinase; protein kinase C

Human mast cells (HMC-1^{560} and HMC-$1^{560, 816}$) can be found in patients with different kinds of mastocytosis. Both cell lines express the receptor TyrK KIT in their membrane. KIT is composed of an extracellular and an intracellular part. In the extracellular part the ligand-binding site is situated with five immunoglobulin likes regions, three of them are involved in ligand binding. The natural ligand of the KIT receptor is the stem cell factor (SCF). SCF binding results in proliferation and in inhibition of apoptosis [Linnekin, 1999]. The intracellular part of the receptor TyrK KIT consists of a juxtamembrane region and two Tyr kinases separated by a kinase insert. In HMC-1 cells KIT is constitutive activated [Sundstrom et al., 2003]. This activation is caused by mutations in the proto-oncogene c-kit, what encodes the KIT protein. These lead to an amino acid exchange in the protein, on the one hand in Gly-560 → Val (HMC-1^{560}), and on the other Asp-816 → Val (HMC-$1^{560, 816}$). The change in the position 560 occurs in the juxtamembrane region of KIT and results in the permanent ligand independent activation of KIT in both cell lines. The mutation is common in various human malignant diseases, including gastrointestinal stromal tumour and cutaneous

Abbreviations used: HMC-1, human mast cell line; TyrK, tyrosine kinase; SCF, stem cell factor; Ca^{2+}, calcium; NH_4Cl, ammonium chloride.

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mastocytosis [Shah et al., 2006]. HMC-$1^{560, 816}$ cells carry both mutations. The Asp-816 → Val change occurs in the intracellular part of KIT and modifies the conformation of the TyrK [Ma et al., 2002; Yavuz et al., 2002; Akin et al., 2003].

Mastocytosis is a mast cell disease characterized by an abnormal growth and accumulation in one or more tissues [Valent et al., 2001]. Systemic mastocytosis almost only occurs in adults and is associated with an activating mutation in codon 816 of c-kit. The diagnosis is based on multifocal histological lesions in the bone marrow or other extra-cutaneous organs. The detection of the activating c-kit point mutation at codon 816 is one diagnostic criterion of systemic mastocytosis. The mutation could be identified in more than 80% of systemic mastocytosis cases [Akin and Metcalfe, 2004; Garcia-Montero et al., 2006]. Especially in its aggressive variants and in mast cell leukaemia, the 816 mutation of KIT seems to play an important role [Valent et al., 2001]. Even if this mutation appears in grave diseases, there is not much known about activating routes in HMC-$1^{560, 816}$ cells.

The TyrK inhibitor STI571 (Glivec®) is very successfully used in cells that carry the 560 mutation. The drug binds with a high affinity to the inactive conformation of the intracellular kinase of the receptor and keeps it in this condition. STI571 acts as a competitive inhibitor of adenosine triphosphate (ATP) on its binding site [Shah et al., 2006]. Thereby it is able to inhibit proliferation and to induce apoptosis of the permanent activated cells [Heinrich et al., 2000]. Unfortunately, it is ineffective in cells with Asp816Val mutation that are in the majority of aggressive systemic mastocytosis and mast cell leukaemia [Valent et al., 2001; Ma et al., 2002]. This behaviour towards STI571 is due to its confirmation change that prevents drug binding on the TyrK receptor.

In both cell lines, it is demonstrated that the constitutive KIT phosphorylation activated other transduction pathways like that of phosphatidylinositol-3′-kinase (PI3K) and mitogene activated protein kinase (MAPK). JAK/STAT and Scr signalling pathways are also associated with KIT and thereby involved in cell survival and proliferation. They are considered to be as well permanent activated in both HMC-1 lines [Linnekin, 1999; Sundstrom et al., 2003].

We have previously observed that long-term pre-incubation of HMC-1^{560} cells with STI571 affects histamine release (manuscript in press). Non-stimulated cells released less histamine, which is attributable to the inhibition of subsequent pathways that are connected with KIT. Cells stimulated by alkalinisation increased their transmitter release. We concluded that STI571 could act as well on other tyrosine kinases than KIT and that proliferation and exocytotic processes have different transduction pathways.

To evidence our thesis, in this work we performed histamine assays with STI571 in HMC-$1^{560, 816}$ cells that different authors have described to be resistant to this drug. We stimulated them with NH_4Cl and the Ca^{2-} ionophore ionomycin and compared the results with those of HMC-1^{560}.

The aim of this work was to study the influence of the KIT mutation in signalling pathways in two HMC-1 sublines. This is important since these cells appear in different kinds of mastocytosis. Therefore, a better understanding of both cellular lines and the knowledge of signalling pathways involved in their activation could lead to a more efficient drug targeting.

METHODS

Chemicals

Ammonium chloride (NH_4Cl) was from Panreac (Barcelona, Spain); rottlerin, ionomycin, Gö6976, GF109203X, genistein and lavendustin A were from Alexis Corporation (Läufelfingen, Switzerland); 2,7-bis (carboxyethyl)-5(6)carboxyfluorescein-acetoxymethylester (BCECF AM) and FURA-2 AM were from Molecular Probes (Leiden, The Netherlands). Phorbol 12-myristate 13-acetate (PMA), Cromoglycic acid and MTT (3-[4,5-dimethyldiazol-2-yl]-2,5-diphenyltetrazolium bromide) were from Sigma–Aldrich (Madrid, Spain). STI571 was provided by Dr. Luis Escribano Mora (Servicio de Hematología, Hospital Ramón y Cajal, Madrid).

Cell Cultures

HMC-1^{560} cells were kindly provided by Dr. J. Butterfield (Mayo Clinic, Rochester, MN) and HMC-$1^{560, 816}$ cells were kindly provided by Dr. Luis Escribano Mora with permission from Dr. J. Butterfield. They were maintained in

Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells were expanded weekly.

Cell Preparation

For histamine release assays, cells were centrifuged (1,500 rpm, 5 min, 4°C) and washed twice with saline solution (1,000 rpm, 5 min, 4°C). The composition of this solution was (mM): Na^- 142.3; K^- 5.94; Ca^{2-} 1; Mg^{2-} 1.2; Cl^- 126.2; HCO_3^- 22.85; HPO_4^{2-} 1.2, SO_4^{2-} 1.2; glucose 1 g/l.

For Ca^{2-} and pH measurements cells were treated in the same conditions, but washed in saline solution plus 0.1% bovine serum albumin (BSA).

The incubation medium was equilibrated with CO_2 prior to use. During the experiments, pH (7.20) was maintained constant by bubbling CO_2. Experiments were carried out at least three times, by duplicate, both for histamine release assays and Ca^{2-} and pH measurements.

Cell Incubation

6.2 μl of a freshly prepared concentrated solution of each drug were added to the incubation medium to attain a final volume of 150 μl and pre-incubated. When the medium reached 37°C, 100 μl of a cell suspension with an approximate density of $1.5–2 \times 10^6$ cells/ml were added to each tube. Incubations were carried out in a bath at 37°C for 10 min.

The incubations were stopped by immersing the tubes in a cold bath. After centrifugation at 2,300 rpm for 10 min, the supernatants were collected and decanted into other tubes for histamine determination. Appropriate controls to determine spontaneous histamine release in the absence of stimuli were executed in every experiment.

Histamine Release Assays

Histamine release was tested with a spectrofluorometer, (Fluoroskan II, Labsystems, Barcelona, Spain) in both pellet (non-released histamine) and supernatant (released histamine) according to Shore's method [Shore, 1971]. To form the fluorescent complex 0.04% orthophthaldialdehyde was used, also trichloroacetic acid (14%) to avoid protein interferences in the histamine release determination. To ensure total histamine, pellets were sonicated for 60 s in 0.2 ml of 0.1 N HCl. In NH_4Cl experiments, histamine release was measured only in pellets, since this compound interferes with the fluorescent complex. Results shown were expressed as the percentage of released histamine from the total histamine content.

Cell Viability

After exposure to different concentrations of STI571 during 24 h in culture medium, cells were centrifuged (1,500 rpm, 5 min, 4°C). The pellets were resuspended in saline solution with MTT (250 μg/ml) and incubated at 37°C for 30 min in darkness.

After washing twice with saline solution cells were sonicated in water for 60 s. The coloured formazan salt was measured at 595 nm in a spectrophotometer plate reader.

Measurement of Cytosolic Free Ca^{2-} and Intracellular pH

HMC-1 cells were loaded with FURA-2 AM (0.2 μM) and BCECF AM (0.05 μM) in a bath at 37°C, for 10 min. After this time, loaded cells were washed with saline solution (1,000 rpm, 10 min, 4°C). Cells were attached to glass coverslips treated with poly-L-lysine, and these were inserted into a thermostated chamber (Life Sciences Resources, UK). Cells were viewed using a Nikon Diaphot 200 microscope equipped with epifluorescence optics (Nikon 40×—immersion UV—Fluor objective). Addition of drugs was made by aspiration and addition of fresh bathing solution to the chamber. Intracellular pH and cytosolic Ca^{2-} concentration were obtained from the images collected by fluorescence equipment (Life Sciences Resources). The light source was a 175 W xenon lamp, and the wavelengths were selected with filters. For FURA-2 AM, the excitation wavelengths were 340 and 380 nm, with emission at 505 nm; for BCECF AM the excitation was performed at 440 and 490 nm, with 530 nm for emission. The calibration of the fluorescence values versus intracellular Ca^{2-} was made according to the method of Grynkiewicz et al. [1985] and the calibration of fluorescence values versus pH as per Thomas et al. [1979]. In brief, a calibration curve was obtained with four known values of pH, measuring the fluorescence ratio obtained in the presence of nigericin, in a K^- solution, for each pH value.

Statistical Analysis

Results were analysed using the Student's *t*-test for unpaired data. A probability level of 0.05 or smaller was used for statistical significance. Results were expressed as the mean ± SEM.

RESULTS

In this work, we checked the release of histamine, intracellular Ca^{2-} and pH changes in HMC-$1^{560, 816}$ in the presence of stimuli.

We have previously described that alkalinisation induces histamine release in HMC-1^{560} in a dose-dependent manner [Pernas-Sueiras et al., 2005]. We showed that intracellular pH increases after NH_4Cl addition without changing cytosolic Ca^{2-} concentrations. Now we treated HMC-$1^{560, 816}$ cells in the same way and could observe a dose-dependent activation of exocytosis (Fig. 1A). Histamine release increased with increasing NH_4Cl concentrations. Further, we observed intracellular pH and Ca^{2-} after adding different concentrations of NH_4Cl. As Figure 1B shows, pH increases immediately after drug addition, where at the highest concentration (100 mM) induced the highest alkalinisation (pH 8.2) and the lowest (20 mM) caused a more slightly increase (pH 7.9). After an initial peak, cytosolic pH

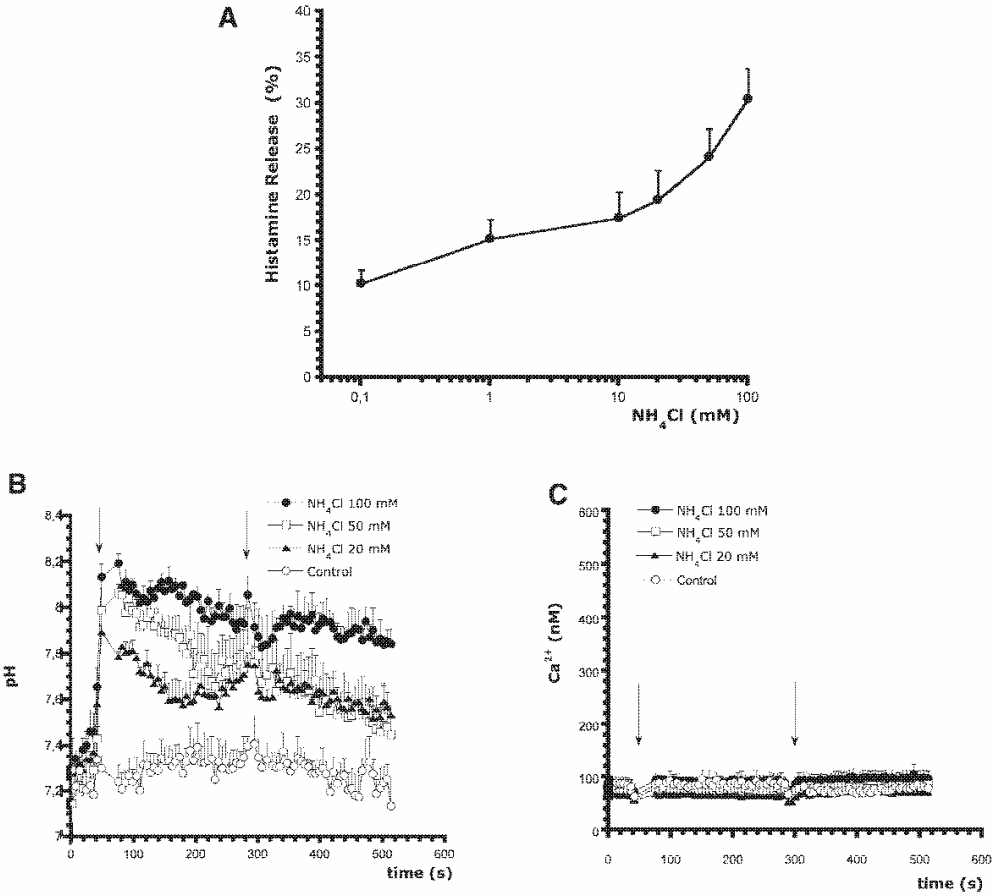

Fig. 1. Effect of NH_4Cl in HMC-$1^{560, 816}$ cells. **A**: Dose–response of histamine released in HMC-$1^{560, 816}$ cells in presence of NH_4Cl. Different concentrations of NH_4Cl were added and histamine release was measured after 10 min of incubation in saline solution. **B**: Variation of cytosolic Ca^{2+} levels in the presence of 20, 50, 100 mM NH_4Cl. The first arrow indicates the addition of NH_4Cl and second that Ca^{2+} (1 mM) was added. **C**: Intracellular pH profile in cells subject to the protocol described in (B). Mean ± SEM of four experiments.

slowly decreased. The experiments were initiated in Ca^{2-} free medium and later on Ca^{2-} was restored to the extracellular medium. Figure 1C shows that Ca^{2-} addition did not affect the declining pH. None of the NH_4Cl concentrations modified intracellular Ca^{2-} levels. During the whole experiment, cytosolic Ca^{2-} concentration remained constant at a baseline.

The Ca^{2-} ionophore ionomycin is another reported stimulus for histamine release in HMC-1[560]. The stimulation is based on a dose-dependent intracellular Ca^{2-}-increase combined with pH-increase. In HMC-1[560, 816] we found as well a release of histamine depending on the dose of ionomycin that was added (Fig. 2A). At the point where ionomycin was added (Fig. 2B), intracellular Ca^{2-} concentration increased rapidly due to the release of the cation from intracellular reservoirs. Subsequent, cytosolic Ca^{2-} dropped down fast to the baseline concentration. The following addition of Ca^{2-} (1 mM) to the medium induced a second intracellular Ca^{2-} increase, due to the influx of the ion from extracellular. Figure 2B shows that intracellular Ca^{2-} reached higher levels with higher concentrations of ionomycin, both in the depletion of intracellular reservoirs (first peak), and in a similar trend when Ca^{2-} enters from the extracellular medium (second peak). Observing pH levels, Figure 2C, in the same experiments, pH baseline moved around 7.2 and increased after adding Ca^{2-}.

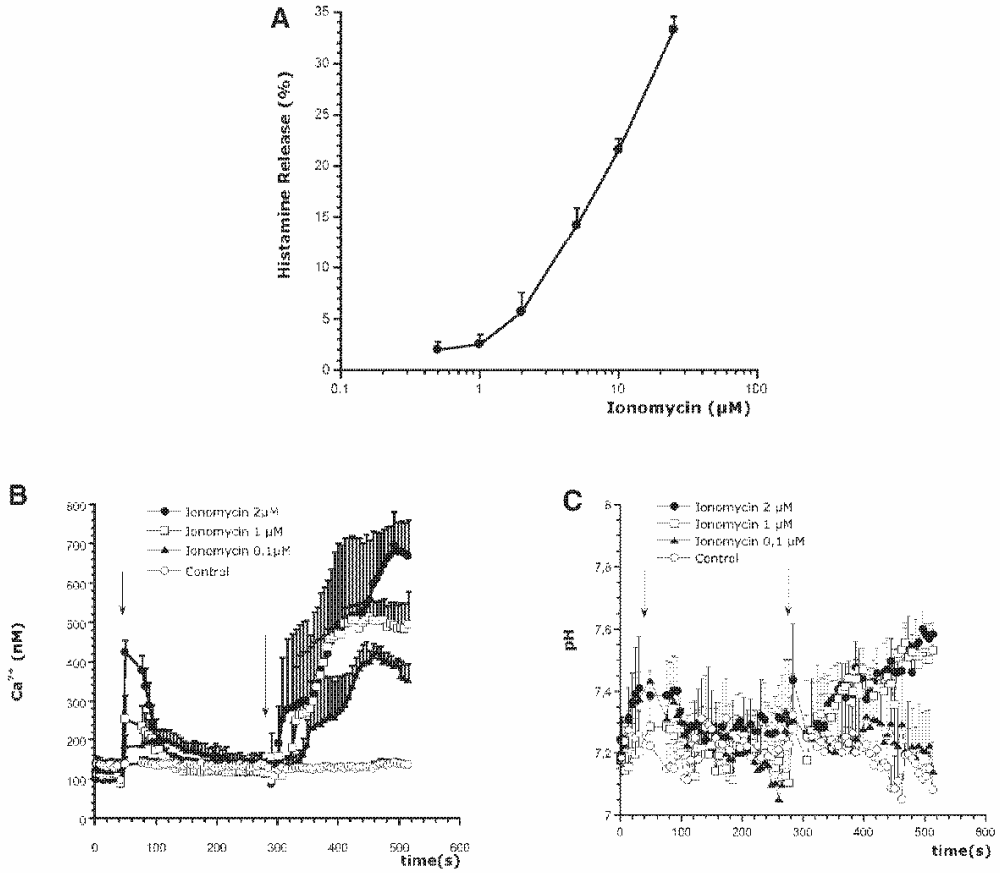

Fig. 2. Effect of ionomycin in HMC-1[560, 816] cells. **A**: Dose–response of histamine released in HMC-1[560, 816] cells in presence of ionomycin. Different concentrations of ionomycin were added and histamine release was measured after 10 min of incubation in saline solution. **B**: Variation of cytosolic Ca^{2+} levels in the presence of 0.1, 1, 2 μM ionomycin. The first arrow indicates the addition of NH_4Cl and second that Ca^{2+} (1 mM) was added. **C**: Intracellular pH profile in cells subject to the protocol described in (B). Mean ± SEM of four experiments.

We examined also the influence of STI571 after long-time incubation in HMC-$1^{560, 816}$ cells. Up to now, it is reported that this drug does not have effects in this cell line.

First, we tested the viability of the cells in the presence of different concentrations of STI571. As Figure 3 shows, in contrast to HMC-1^{560}, HMC-$1^{560, 816}$ cells did not die when they were exposed to the TyrK inhibitor in concentrations between 0.1 nM and 10 μM.

Furthermore, we incubated HMC-$1^{560, 816}$ cells with 25 nM during 24 h in culture medium and performed afterwards histamine assays. We selected this concentration from previous experiences in HMC-1^{560} cells. Surprisingly, spontaneous histamine release was significantly decreased in STI571 24 h pre-incubated HMC-$1^{560, 816}$ cells (Fig. 4). This result gave us reason to investigate more the behaviour of these cells concerning to long-time incubation with STI571 and release of histamine. We stimulated them with NH_4Cl (50 mM) after 24-h pre-incubation with STI571 (25 nM). As Figure 5A shows, pre-treated cells released more histamine than control cells after alkalinisation. In contrast, HMC-$1^{560, 816}$ cells did not change their histamine response when they were pre-incubated with STI571 and further stimulated with ionomycin (2 μM), compared to ionomycin treated cells (Fig. 5B).

In HMC-1^{560}, the effect of NH_4Cl was due to a higher increase of pH in cells pre-incubated with the TyrK inhibitor after alkalinisation. To investigate this in HMC-$1^{560, 816}$, intracellular was measured after 24 h STI571 (25 nM) pre-treatment. In Figure 6A, it is shown that pH_i reached higher values in STI571 pre-incubated HMC-$1^{560, 816}$ cells, whereas intracellular Ca^{2-}

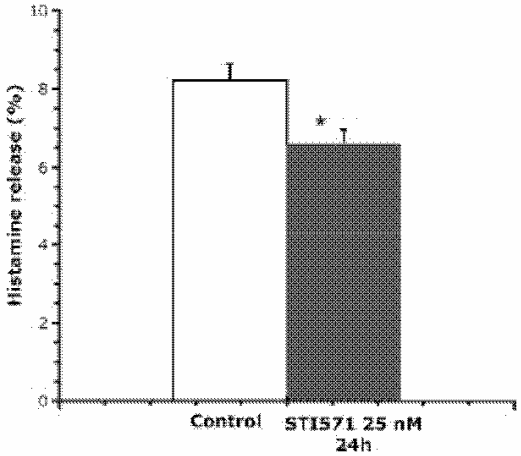

Fig. 4. Effect of STI571 on HMC-$1^{560, 816}$ spontaneous histamine release after 24 h of incubation. Cells were pre-incubated for 24 h in the presence of STI571 (25 nM) in culture medium. Histamine release was checked after 10 min of incubation in saline solution at 37°C. Mean ± SEM of four experiments. (*) Significant differences between control and drug pre-incubated cells.

did not change at any moment of the experiments (Fig. 6B).

From previous studies, we know that PKC stimulation by the phorbol ester PMA increases the release of histamine after NH_4Cl stimulation in STI571 pre-incubated HMC-1^{560} cells (manuscript in press). As Figure 7A shows, STI571 increased significantly alkalinisation-induced histamine release after treatment with PMA (100 ng/ml) in HMC-$1^{560, 816}$. However, we could not observe any differences neither in cytosolic Ca^{2-}, nor in pH levels comparing STI571 pre-incubated cells with control cells

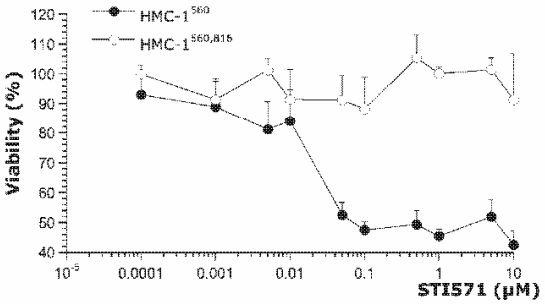

Fig. 3. HMC-1^{560} and HMC-$1^{560, 816}$ viabilities after 24 h incubation with STI571. Different concentrations of STI571 were added in culture medium and cells were incubated for 24 h at 37°C. Cell viability was checked by MTT test. Control cells with non-STI571 treatment, were used as 100% viability and ethanol treatment as 100% dead, in each experiment. Mean ± SEM of four experiments.

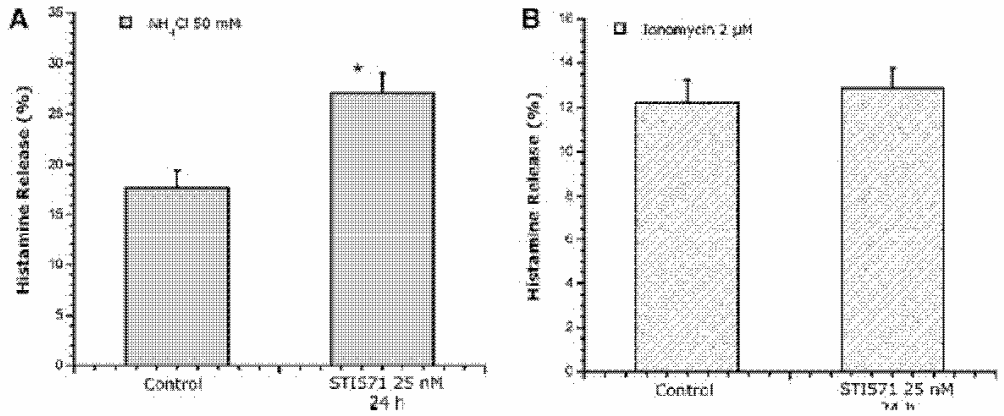

Fig. 5. Effect of STI571 on alkalinisation- and ionomycin-induced histamine release in HMC-1$^{560, 816}$ cells. STI571 (25 nM) 24 h pre-incubated cells were stimulated with NH_4Cl (50 mM) (**A**) or ionomycin (2 µM) (**B**) during 10 min in saline solution at 37°C and histamine release was measured. Mean ± SEM of four experiments. (*) Significant differences between control and drug pre-incubated cells.

(data not shown). There was no effect when STI571 pre-treated cells were stimulated with ionomycin (2 µM) in combination with PMA (100 ng/ml) (Fig. 7B).

When PKC was inhibited by GF109203X (500 nM), 24-h pre-incubation with STI571 did not modify histamine release neither in cells stimulated by NH_4Cl (50 mM) (Fig. 8A), nor by ionomycin (0.1 µM) (Fig. 8B). HMC-1$^{560, 816}$ cells with PKC inhibited by Gö6976 (100 nM) showed the same behaviour (Fig. 8C,D). In contrast, as Figure 9A shows, when PKC was inhibited by rottlerin (10 µM) and subsequently NH_4Cl (50 mM) stimulation took place, surprisingly STI571 pre-treated cells enhanced histamine release. That meant that when TyrK and PKC were inhibited the effect on histamine release was potentiated. We could not observe any difference comparing STI571 and control cells, when they were treated with rottlerin (10 µM) and afterwards stimulated with ionomycin (Fig. 9B). In continuation, we performed rottlerin experiments in the fluorescent microscope, shown in Figure 10. HMC-1$^{560, 816}$ cells were pre-incubated with STI571 (25 nM) during 24 h like in histamine assay. We began the experiments in Ca^{2-}-free medium. After obtaining a baseline, we added NH_4Cl (50 mM). As

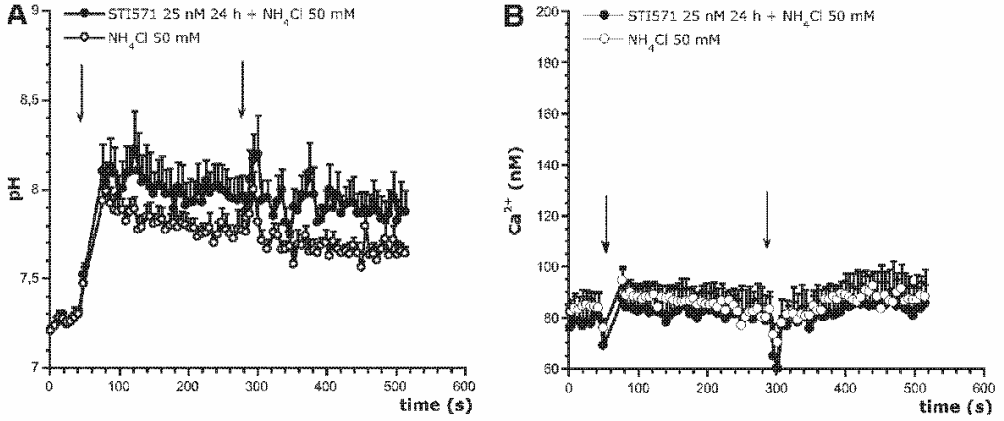

Fig. 6. Effect of STI571 on intracellular Ca^{2+} and pH levels in HMC-1$^{560, 816}$ cells. Cells were pre-incubated for 24 h in the presence of STI571 (25 nM) in culture medium. Cytosolic pH (**A**) and Ca^{2+} (**B**) changes were observed. The first arrow indicates the addition of NH_4Cl (50 mM) and second that Ca^{2+} (1 mM) was added. Mean ± SEM of four experiments.

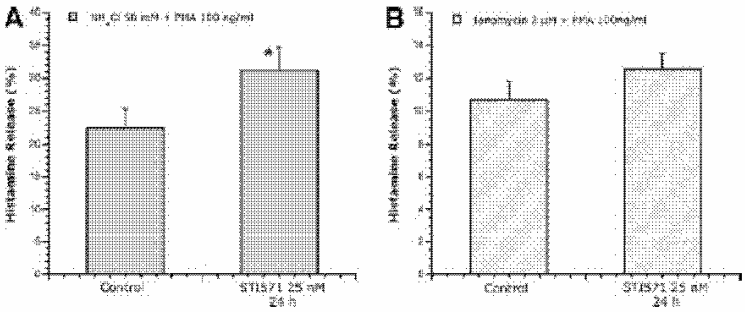

Fig. 7. Effect of PKC activation and STI571 on alkalinisation- and ionomycin-induced histamine release in HMC-1[560, 816]. Cells pre-incubated with STI571 (25 nM) during 24 h were pre-treated with PMA (100 ng/ml) 10 min and afterwards stimulated with NH_4Cl (50 mM) (**A**) or ionomycin (2 μM) (**B**) during 10 min in saline solution at 37°C and histamine release was measured. Mean ± SEM of four experiments. (*) Significant differences between control and drug pre-incubated cells.

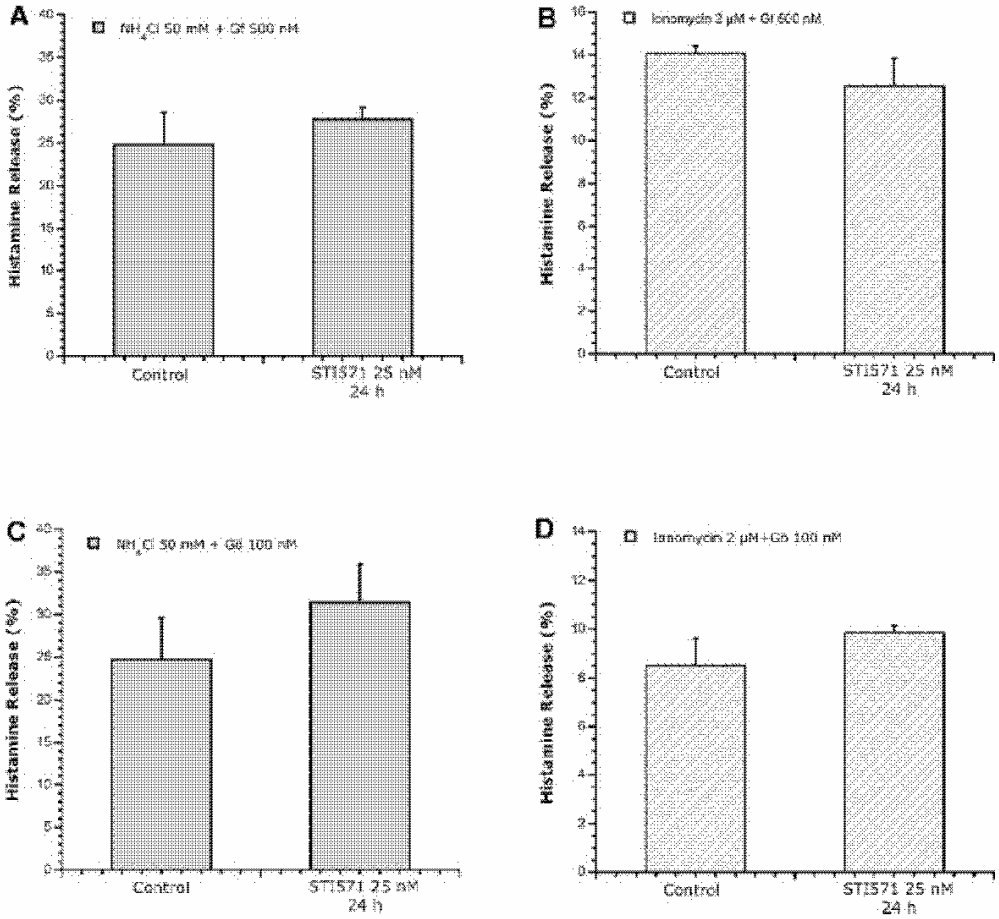

Fig. 8. Effect of PKC inhibition and STI571 on alkalinisation- or ionomycin-induced histamine release in HMC-1[560, 816]. Cells pre-incubated with STI571 (25 nM) during 24 h were pre-treated with Gö6976 (100 nM) and GF109203X (500 nM) 10 min and afterwards stimulated with NH_4Cl (50 mM) (**A**,**C**) or with ionomycin (2 μM) (**B**,**D**) during 10 min in saline solution at 37°C and histamine release was measured. Mean ± SEM of four experiments.

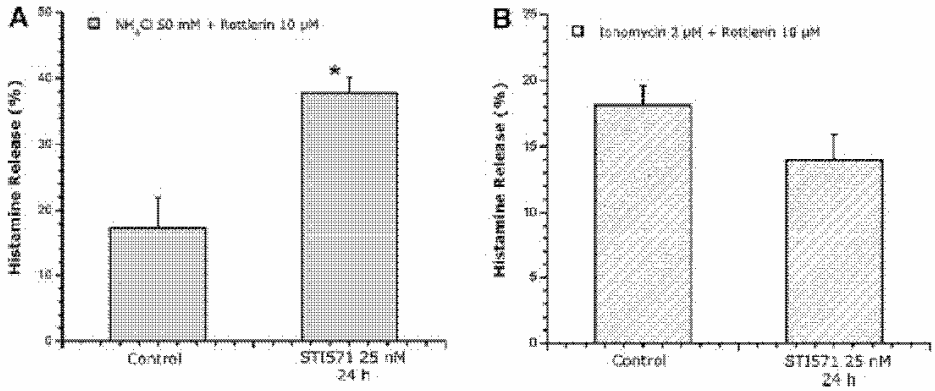

Fig. 9. Effect of rottlerin and STI571 on alkalinisation- or ionomycin-induced histamine release in HMC-$1^{560, 816}$. Cells pre-incubated with STI571 (25 nM) during 24 h were pre-treated with rottlerin (10 µM) 10 min and afterwards stimulated with NH_4Cl (50 mM) (**A**) or with ionomycin (2 µM) (**B**) during 10 min in saline solution at 37°C and histamine release was measured. Mean ± SEM of four experiments.

Figure 10 shows, pH increased significantly more in STI571 pre-treated cells at the point of NH_4Cl addition than in control cells. After adding Ca^{2-} (1 mM) to the medium, pH seemed to elevate lightly again.

DISCUSSION

It is described in previous reports that NH_4Cl induces alkalinisation and no Ca^{2-} increase in rat mast cells [Alfonso et al., 2000]. In these cells, it was suggested for the first time that pH increase by itself could be an enough signal to induce histamine release. Studies in HMC-1 have clearly demonstrated that they also show this behaviour after alkalinisation. Exocytotic process takes place without affecting intracellular Ca^{2-} concentrations [Pernas-Sueiras et al., 2005, 2006a,b]. In those studies, we used HMC-1^{560} cells that carry a mutation in the proto-oncogene c-kit that leads to a Glycin → Valin amino acid exchange in position 560 of the receptor TyrK KIT.

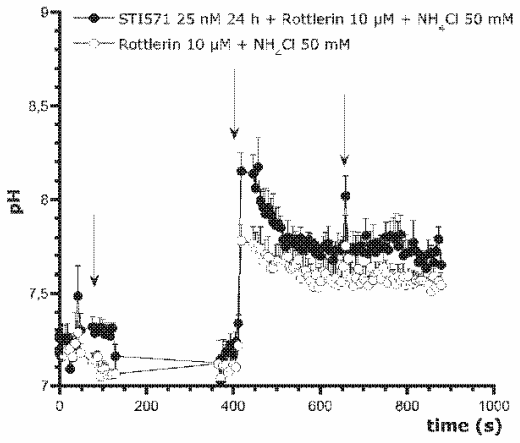

Fig. 10. Effect of rottlerin and STI571 on alkalinisation-modulated intracellular pH levels in HMC-$1^{560, 816}$ cells. Cells were pre-incubated for 24 h in the presence of STI571 (25 nM) in culture medium. Cytosolic pH changes were observed. The first arrow indicates the addition of rottlerin (10 µM), the second of NH_4Cl (50 mM) and the third that Ca^{2+} (1 mM) was added. Mean ± SEM of three experiments.

In the present work, the HMC-$1^{560, 816}$ subline with one amino acid change more in position 816 was used to check if its behaviour is similar regarding release of histamine after stimulation. This is important since there are already differences reported between both sublines [Sundstrom et al., 2003; Gleixner et al., 2005].

Results shown confirmed the same dose-dependent histamine release in presence of NH_4Cl in HMC-$1^{560, 816}$ like in cells with one mutation. We also demonstrated that intracellular alkalinisation took place without changing Ca^{2-} concentrations. However, pH increased much more in HMC-$1^{560, 816}$ than in HMC-1^{560} after alkalinisation. When HMC-$1^{560, 816}$ cells were treated with 50 mM of NH_4Cl, pH increased about 0.8 units; in contrast, treating HMC-1^{560} with the same concentration, pH rose only about 0.3 units higher, whereas histamine release was almost the same. We suggest that pH control has a larger importance in HMC-$1^{560, 816}$ than in HMC-1^{560},

but more studies have to be addressed in this context.

The Ca^{2-} ionophore ionomycin is another stimulus reported to induce exocytosis in HMC-1^{560} [Pernas-Sueiras et al., 2005] and other mast cells [Teofoli et al., 1999]. In HMC-1^{560} the stimulation is due to an increase of intracellular Ca^{2-} concentration in combination with an alkalinisation in presence of extracellular Ca^{2-}. We could demonstrate that histamine release can be stimulated with ionomycin in a dose-dependent manner, also in HMC-$1^{560, 816}$. We saw that HMC-$1^{560, 816}$ cells did not reach a plateau at concentrations up to 10 μM of ionomycin as HMC-1^{560}, comparing both histamine release profiles [Pernas-Sueiras et al., 2005]. Other differences were shown in intracellular Ca^{2-} observations. After ionomycin addition, cytosolic Ca^{2-} concentration increased due to the release of the cation from intracellular reservoirs. This raise was more steeply but reached lower values in HMC-$1^{560, 816}$ than in HMC-1^{560}. When Ca^{2-} entered from the extracellular medium, intracellular Ca^{2-} concentration increased less and more slowly in cells with two mutations. The presence of the second mutation modifies the regulatory mechanisms for calcium.

Differences in the two sublines apart from the mutations have been already described. HMC-$1^{560, 816}$ has a higher proliferation rate in culture, whereas the cells are smaller and more homogenous. HMC-1^{560} cells are heterogeneous in size and showed homotypic aggregation. HMC-$1^{560, 816}$ has a normal diploid DNA profile but HMC-1^{560} indicate an increased DNA content [Sundstrom et al., 2003]. However, one of the most interesting differences is the response of the two cell lines in presence of KIT TyrK inhibitor STI571. Whereas STI571 induces apoptosis and inhibits proliferation in HMC-1^{560} cells, it does not affect HMC-$1^{560, 816}$ cells due to the conformation change in the kinase of KIT, that avoids drug binding [Heinrich et al., 2000; Ma et al., 2002; Akin et al., 2003; Roskoski, 2003].

Long-time incubation of HMC-1^{560} cells with STI571 modifies histamine release, spontaneous release decreases and alkalinisation-induced histamine release increased, caused by a pH_i increase to higher values after NH_4Cl addition (manuscript in press). STI571 could inhibit other Tyr kinases, and in this study, we showed that STI571 affected histamine release in HMC-$1^{560, 816}$ similarly after long-time incubation. This suggests that STI571 might have other binding sites. STI571 is reported to bind the ATP binding site of the KIT kinase in HMC-1^{560} [Roskoski, 2003]. The drug is not able to ligate the same site in HMC-$1^{560, 816}$, because of the conformation change in the KIT kinase caused by the amino acid exchange in position 816 [Ma et al., 2002; Sundstrom et al., 2003]. If the drug would selectively inhibit KIT TyrK, we could not observe similar effects in HMC-1^{560} and HMC-$1^{560, 816}$ cells. We have suggested that inhibition takes place in other Tyr kinases, since we found that lavendustin A and genistein, two unselective TyrK, were also able to reduce spontaneous histamine release after long-time incubation HMC-1^{560} (manuscript in press). The two drugs induced also an increase in alkalinisation-induced exocytosis. This might be through Src Tyr kinases, since it is reported that their inhibition by genistein avoids intracellular pH increase by inhibiting an anion Cl^-/HCO_3^- exchanger in cardiomyocytes [Puceat et al., 1998].

Our cell viability test and studies of other pharmacological groups demonstrate clearly that KIT TyrK inhibition takes place in HMC-1^{560} but not in HMC-$1^{560, 816}$ [Heinrich et al., 2000; Akin et al., 2003]. Therefore proliferation and histamine release might have completely unconnected signalling pathways. This agrees with other studies that have reported that Ras-MAPK-ERK and PI3K pathways do not seem to be essential for exocytosis in this cell line [Kempna et al., 2004]. These pathways are directly connected with KIT and essential for proliferation [Linnekin, 1999]. In contrast, regarding histamine release, pH seems to play a mayor role in HMC-1, as it was reported in rat mast cells [Vilarino et al., 1999; Alfonso et al., 2000]. Modulation of pH can be due to the activity of anion Cl^-/HCO_3^- [Puceat et al., 1998] or cation H^-/Na^- [Alfonso et al., 1994, 1998; Friis and Johansen, 1996] exchanger.

It has been described that STI571 act very selective on KIT TyrK [Heinrich et al., 2000]. The selectivity of the compound should be studied in other enzymes and ion exchangers, since pH is influenced after long-term drug incubation. It should also be bear in mind that HMC-$1^{560, 816}$ are more sensitive to extracellular pH changes.

Our results show that STI571 does not modify ionomycin-induced histamine release in cells

with one or two mutations. It seems that the pathway that is modified by STI571 is not linked to that of ionomycin and probably not to intracellular Ca^{2-} concentrations. Until now, we could never observe that cytosolic Ca^{2-} has a different profile in STI571 pre-incubated cells.

Finally, we checked the influence of PKC modulation on STI571 effects in HMC-$1^{560, 816}$ since we had earlier reported that activation of PKC by PMA increased histamine response after alkalinisation in STI571 pre-treated HMC-1^{560}cells (manuscript in press). We found the same behaviour of HMC-$1^{560, 816}$ cells.

Surprisingly, STI571 pre-incubated HMC-$1^{560, 816}$ cells with rottlerin inhibited PKC, released more histamine after alkalinisation. This effect might be linked to the fact that pH rose to higher values in STI571 cells. Since the TyrK inhibitor was discovered by testing of compounds for inhibition of PKC [Shah et al., 2006]. Maybe this potentiated effect is due to a common binding site of the two inhibitors that modify pH regulation in HMC-$1^{560, 816}$. Nevertheless, as far as we know, it has not been reported that PKC inhibition induced histamine release in mast cells. In contrast, we could not observe an effect of PKC inhibition in HMC-1^{560}. The difference could be due to the higher pH sensitivity of HMC-$1^{560, 816}$.

More studies should be made in HMC-$1^{560, 816}$ concerning to activating processes and the influence of the mutations in this context especially in pH modulation. Much work is already done in HMC-1^{560} and this is a good starting point to compare the two sublines in the future. This is important because both lines appear in different diseases and drug targeting could be more efficient with a better knowledge of intracellular signalling.

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3.2 SECCIÓN II

II.A: Role of Extracellular HCO_3^- in Cytosolic pH Regulation and Cell Viability of HMC-1 Human Mast Cells

Role of extracellular HCO_3^- in cytosolic pH regulation and cell viability of Human Mast Cells HMC-1

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Key words: HMC-1, mast cells, acidification, intracellular pH, Na^+ transport, HCO_3^- transport

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ABSTRACT

The role of external Na^+ and HCO_3^- on intracellular pH (pH_i) and their regulation by HCO_3^- transporters and the Na^+/H^+ exchanger (NHE1) was studied in Human Mast Cell lines (HMC-1). HMC-1^{560} and HMC-$1^{560,816}$ cells have activating mutations in the proto-oncogene c-kit that cause autophosphorylation and permanent KIT tyrosine kinase activation. These mutations lead to a Gly-560 -> Val exchange in both sublines and to an Asp-816 -> Val amino acid change in HMC-$1^{560, 816}$ cells.

HCO_3^- and CO_2 comprise the main buffering mechanism implicated in cytosolic pH regulation. In both HMC-1 sublines pH_i immediately decreased in HCO_3^- free medium. Further the pH_i fall was even more evident in Na^+/HCO_3^- free medium. Intracellular pH was stabilized when the external pH was elevated or after addition of different concentrations of $NaHCO_3$ but not $KHCO_3$. The carbonic anhydrase inhibitor acetazolamide retarded pH_i decrease in HCO_3^- free medium rather the anion exchanger inhibitor NPPB and the inhibitor of the Na^+/H^+ exchanger EIPA forced the pH_i fall in HMC-$1^{560,816}$ cells.

Further short time incubation of HMC-$1^{560,816}$ cells in HCO_3^- or Na^+/HCO_3^- free medium significantly decreased cell viability, whereas HMC-1^{560} cells were not affected. Long time incubation with different anion exchanger inhibitors (DIDS, NPPB) decreased viability of both sublines.

These results demonstrate the existence of a strong Na^+/HCO_3^- dependent pH regulating mechanism which is connected to NHE1 in HMC-1 cells. Either external HCO_3^- as well as functioning of HCO_3^- exchange is important for cell viability.

Abbreviations: HMC-1: Human Mast Cell Line; NH_4Cl: ammonium chloride; HCO_3^-: bicarbonate; pH_i: intracellular pH

INTRODUCTION

Human mast cell lines (HMC-1^{560} and HMC-$1^{560,816}$) were obtained from a patient with mast cells leukaemia (Butterfield et al., 1988), and patients with different kinds of mastocytosis express a similar type of cells. Both cell lines express in their membrane the constitutive activated TyrK receptor KIT (Sundstrom et al., 2003). This activation is caused by mutations in the proto-oncogene c-kit, which encodes the KIT protein. These leads to an amino acid exchange in the protein, on the one hand in Gly-560->Val (HMC-1^{560}), and on the other Asp-816->Val (HMC-$1^{560,816}$). The change in the position 560 occurs in the juxtamembrane region of KIT and results in the permanent ligand independent activation of both sublines. The mutation is common in various human malignant diseases, including gastrointestinal stromal tumour and cutaneous mastocytosis (Shah et al., 2006). HMC-$1^{560,816}$ cells carry both mutations. The Asp-816->Val change occurs in the intracellular part of KIT and modifies the conformation of the tyrosine kinase (Akin et al., 2003; Ma et al., 2002; Yavuz et al., 2002). The mutation could be identified in more than 80% of systemic mastocytosis cases (Akin and Metcalfe, 2004; Garcia-Montero et al., 2006).

Intracellular pH (pH_i) plays an important role in the activation of these cells. NH_4Cl addition dose-dependently increased pH_i without modifying cytosolic Ca^{2+} concentrations. Further histamine release could be triggered with just an intracellular alkalinisation and no Ca^{2+} increase. On the other hand alkaline induced exocytosis was modulated by PKC and cAMP activators and tyrosine kinase inhibitors (Lober et al., 2008a; Lober et al., 2008b; Pernas-Sueiras et al., 2005; Pernas-Sueiras et al., 2006a; Pernas-Sueiras et al., 2006b). Upon these results it seemed to be necessary to understand how intracellular pH is regulated in HMC-1^{560} and HMC-$1^{560,816}$ cells and which H^+ and HCO_3^- transporters are expressed in these cells.

The Na^+/H^+ exchanger 1 (NHE1) is activated during intracellular acidification and translates sodium from extracellular against intracellular protons. Its activation can lead to alkalinisation. NHE1 is ubiquitously

expressed in the integral plasma membrane of almost all cell types and it is inhibited by amiloride and its derivates. The exchanger is involved in growth, differentiation, migration, sodium flux and cell volume (De Vito, 2006; Fliegel, 2005; Slepkov et al., 2007). NHE1 is suggested to be an important sodium influx mechanism in rat mast cells and it regulates intracellular pH as well as regulatory volume increase (RVI) (Botana et al., 1992; Cabado et al., 1993). After an artificial acidification rat mast cell recovery rate is two-fold smaller in amiloride treated cells (Alfonso et al., 1998). Rat mast cells undergo alkalinisation in hypertonic conditions. This could be inhibited by amiloride and lack of extracellular sodium indicating the role of a Na^+/H^+ exchanger. In those studies as well the participation of an anion exchanger could be detected by inhibiting alkalinisation with DIDS (Cabado et al., 2000).

HCO_3^- transporters are often the major pH regulators in animal cells (Romero and Boron, 1999). Besides basal pH_i maintenance they play a role in cell division, migration and transepithelial ion movement. Different HCO_3^- transporters are function-specifically expressed in distinct tissues and act there in their specialized mode. They can be divided in three major groups: Na^+ independent Cl^-/HCO_3^- exchanger (AE), Na^+/HCO_3^- cotransporters (NBC) and Na^+ driven Cl^-/HCO_3^- exchangers (NCBE, BTR1) (Romero and Boron, 1999; Romero et al., 2004). AEs are mainly activated during intracellular alkalinisation and transport HCO_3^- out of the cell in exchange to extracellular Cl^-. Four isoforms are cloned: AE1-AE4 (Romero et al., 2004). AE1 (SLC4A1) is strongly expressed in red blood cells and renal collecting duct of the kidney. It is structurally and physiologically well studied. AE1 is not sensitive to pH_i changes and unaffected by NH_4^+. In contrast AE2 (SLC4A2), which is widely expressed in non-excitable cells, is strongly inhibited by H^+ and pH_i sensitive. Paradoxically it is activated by NH_4^+ despite NH_4^+-mediated alkalinisation. AE3 (SLC4A3) was found in heart and brain tissue analysis. AE4 (SLC4A9) is expressed in renal collecting duct cells and was controversial discussed as a Na^+ dependent or independent anion exchanger due to its amino acid sequence homology to NBCs. AE1-3 bind to carbonic anhydrase II and IV what stimulates

their transporter activity. Na^+/HCO_3^- cotransporters alkalinize intracellular pH by triggering HCO_3^- influx. NBCe1 (SLC4A4) is electrogenic and predominantly expressed in kidney, pancreas, liver and GI tract (Marino et al., 1999; Romero and Boron, 1999; Schmitt et al., 1999). NBCe2 (SLC4A5) was detected in liver, testis, spleen and other tissues. It mediates Cl^- independent Na^+/HCO_3^- cotransport that can be inhibited by DIDS (Sassani et al., 2002). NBCn1 (SLC4A7) is 59% identical with NBCe1. It was found in human retina and later in spinal cord, muscle, thymus, trachea, testis and other tissues (Choi et al., 2000). Functional studies of this transporter in oocytes indicated an electroneutral Cl^- independent Na^+/HCO_3^- transport, insensitive to DIDS but sensitive to amiloride. A posterior study with a rat NBCn1 homologue demonstrated amiloride resistance and a partial sensitivity to DIDS (Choi et al., 2000; Pushkin et al., 1999a; Pushkin et al., 1999b). NDCBE (SLC4A8) is expressed in testis, ovary, brain, bone marrow and other tissues. It has 56% identity with NBCe1 and 76% with NBCn1. It is controversial discussed if the electroneutral transporter requires Cl^- for its function, since Cl^- depletion did not affect Na^+/HCO_3^- cotransport in one study while in another study oocytes expressing NDCBE demonstrated a Na^+ dependent Cl^-/HCO_3^- exchange (Amlal et al., 1999; Grichtchenko et al., 2001). NCBE (SLC4A10) is an electroneutral Na^+/HCO_3^- exchanger expressed in brain, kidney, ileum and other tissues. It requires HCO_3^- and is blocked by DIDS (Damkier et al., 2007). BTR1 (SLC4A11) is a $Na^+/B(OH)_4^-$ transporter and another member from this gene family (Parker et al., 2001). Recently it was found in human kidney, GI tract and brain. Until now little is known about the last two isoforms of the HCO_3^- transporter family.

As mentioned above studies in rat mast cells already report about a Cl^-/HCO_3^- exchanger activity. In rat peritoneal mast cells a Na^+ independent pH_i fall was observed when the external buffer contained HCO_3^-. Removal of extracellular Cl^- reversed Cl^-/HCO_3^- exchange and induced a Na^+ independent alkaline load. Alkalinisation and its pH_i recovery could be inhibited by DIDS and were HCO_3^- dependent (Jensen et al., 1998). Furthermore, it has been shown that rat mast cells undergo

alkalinisation in HCO_3^- free extracellular medium after $NaHCO_3$ addition. The effect was minor adding $KHCO_3$. The increase was inhibited by amiloride and DIDS suggesting that alkalinisation is controlled by NHE and AE regulating mechanisms in these cells (Vilarino et al., 1998).
The aim of this work was to disclose the role of external Na^+ and HCO_3^- on intracellular pH in HMC-1^{560} and HMC-$1^{560, 816}$ cells and to discuss by which transporters regulation takes place. This will help understanding the mechanism that regulate the cellular biology of mast cells in disease.

METHODS

Chemicals

NH_4Cl was from Panreac (Barcelona, Spain); 2,7-bis (carboxyethyl)-5(6)carboxy-fluorescein-acetoxymethylester (BCECF AM) was from Molecular Probes (Leiden, The Netherlands); DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt), SITS (4-acetamido-4'-isothiocyanato-stilbene-2,2'-disulfonic acid), NPPB (5-nitro-2-[3-phenylpropylamino] benzoic acid), EIPA, acetazolamide and MTT (3-[4,5-dimethyldiazol-2-yl]-2,5-diphenyltetrazolium bromide) were from Sigma-Aldrich (Madrid, Spain).

Cell cultures

HMC-1 cells were kindly provided by Dr. J. Butterfield (Mayo Clinic, Rochester, MN) and maintained in Iscove´s modified Dulbecco´s medium (IMDM) supplemented with 10 % fetal bovine serum (FBS), 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were expanded weekly and not more than 40 passages.

Cell preparation

Cells were centrifuged (1500 r.p.m., 5 minutes, 4 ºC) and washed twice with saline solution (1000 r.p.m., 5 minutes, 4 ºC). The composition of this solution (Umbreit) was (mM): Na^+ 142.3; K^+ 5.94; Ca^{2+} 1; Mg^{2+} 1.2; Cl^- 126.2; HCO_3^- 22.85; HPO_4^{2-} 1.2, SO_4^{2-} 1.2; glucose 1 g/l. The composition of the HCO_3^- free solution was (mM): Na^+ 139.19; K^+ 5.94;

Mg^{2+} 1.2; Cl^- 124.94; HPO_4^{2-} 7.7; $H_2PO_4^-$ 7.7; SO_4^{2-} 1.2; glucose 1 g/l. The composition of the Na^+/HCO_3^-- free solution was (mM): K^+ 5.94; Ca^{2+} 1; Mg^{2+} 1.2; choline 143; Cl^- 149.94; SO_4^{2-} 1.2; Tris-base 8; Tris-HCl 41.95; glucose 1 g/l.

For pH measurements cells were treated and washed in Umbreit solution plus 0.1 % bovine serum albumin (BSA). The incubation medium was equilibrated with CO_2 prior to use. During the experiments, pH (7.20) was maintained constant by bubbling CO_2. Experiments were carried out at least three times, in duplicate.

Measurement of intracellular pH

HMC-1 cells were loaded BCECF AM (0.05 µM) in a bath at 37 ºC, for 10 minutes. After this time, loaded cells were washed with saline solution (1000 r.p.m., 10 minutes, 4 ºC). Cells were attached to glass coverslips treated with poly-L-lysine, and these were inserted into a thermostated chamber (Life Sciences Resources, U.K.). Cells were viewed using a Nikon Diaphot 200 microscope equipped with epifluorescence optics (Nikon 40x– immersion UV– Fluor objective). Addition of drugs was made by aspiration and addition of fresh bathing solution to the chamber. Intracellular pH was obtained from the images collected by fluorescence equipment (Life Sciences Resources, U.K.). The light source was a 175 W xenon lamp, and the used wavelengths were selected with filters. For BCECF AM the excitation was performed at 440 and 490 nm, with 530 nm for emission. The calibration fluorescence values *vs.* pH as per Thomas *et al*. (Thomas et al., 1979). In brief, a calibration curve was obtained with four known values of pH, measuring the fluorescence ratio obtained in the presence of nigericin, in a K^+ solution, for each pH value.

Cell viability

After incubation during 30 or 60 minutes in a water bath or 24 hours in a cell incubator, cells were centrifuged (1500 r.p.m., 5 minutes, 4 ºC). The pellets were resuspended in Umbreit solution with MTT (250 µg/ ml) and incubated at 37 °C for 30 minutes in darkness. After washing twice with

saline solution cells were sonicated for 60 seconds. The coloured formazan salt was measured at 595 nm in a spectrophotometer plate reader.

Western blotting

Cells were incubated during 10 min in Umbreit solution or in Na^+/HCO_3^- free solution. Afterwards cells were lysed and membrane lysates were preparated by centrifugation (43.000xg; 30 min). Cells were resuspended in lysis buffer and blotted by reduced SDS-PAGE.

After one hour of blockage with 5% non-fat dry milk the membrane was incubated overnight with anti-NHE1 (BD Biosciences). After four washes with washing buffer the membrane was incubated for two hours with secondary peroxidase-labelled antibody (Amersham). After four washes chemiluminescence was visualized with SuperSignal® West Pico (Pierce). Relative protein expression was calculated in relation to β-tubulin (Sigma-Aldrich) expression for each experiment. Experiments were carried out at least three times.

Statistical analysis.

Results were analyzed using the Student's t-test for unpaired data. A probability level of 0.05 or smaller was used for statistical significance. Results were expressed as the mean ± SEM.

RESULTS

This work studied the role of external HCO_3^- and Na^+ on intracellular pH and viability of HMC-1^{560} and HMC-$1^{560,816}$ cells.

It has been previously described that alkalinisation induces histamine release in HMC-1^{560} and HMC-$1^{560,\ 816}$ cells without modulating cytosolic Ca^{2+} levels (Lober et al., 2008b; Pernas-Sueiras et al., 2005). Therefore it is important to understand pH-regulating processes in these cells. One of the main mechanisms implicated in pH_i regulation is the CO_2/HCO_3^- buffer system. To study this system cells were bathed in different saline solutions without HCO_3^-. As Fig. 1A shows, intracellular pH dramatically

decreased in HMC-1^{560} cells without HCO_3^-, whereas the extracellular pH was kept constant during the whole experiment. The drop was even more significant in cells without Na^+ and HCO_3^- in the external medium reaching values of about 6.7. The same figure shows that control cells in physiologic saline solution containing Na^+ and HCO_3^- kept their pH_i constant. Neither addition of 20mM $NaHCO_3$ nor increasing extracellular pH could intercept the decrease of pH_i (Fig. 1B.) Only when 50mM $NaHCO_3$ were added to the extracellular Na^+/HCO_3^- free saline solution, a slight arrest in pH_i decrease was observed, which was more significantly when 100mM $NaHCO_3$ were added (Fig. 1C).

Performing similar experiments with HMC-$1^{560, 816}$ cells, pH_i also declined without external HCO_3^-, Fig. 2A, but surprisingly when the extracellular pH was increased from 7.2 to 7.4 the intracellular pH immediately stopped its decrease. A similar but more retarded effect had the addition of 20mM $NaHCO_3$ to the HCO_3^- free saline extracellular solution. These results suggest that, in a pH_i steady-state regulation, a HCO_3^- transporter is implicated, since in both HMC-1 sublines pH_i strongly depends on the presence of extracellular HCO_3^-. This transporter seems to be more sensitive to the extracellular conditions in HMC-$1^{560, 816}$, since these cells stabilized pH_i already after addition of $NaHCO_3$ (20mM) while in HMC-1^{560} at least 50mM of the base were necessary. There are three major groups of HCO_3^- transporters of which two of them are dependent on Na^+. To investigate if the observed pH_i fall is Na^+ dependent, experiments were provided with HMC-$1^{560, 816}$ cells in saline solution without Na^+/HCO_3^- and subsequent addition of 20mM $NaHCO_3$ or $KHCO_3$. As Fig. 2B shows, the addition of HCO_3^- alone could not intercept pH_i decrease because only the addition of HCO_3^- in combination with Na^+ but not K^+ led on to pH_i stabilisation. These results indicate the activity of a strongly Na^+ dependent HCO_3^- mechanism in HMC-$1^{560, 816}$ cells. As we could not observe a spontaneous end of pH_i falling in none of the HMC-1 sublines neither in HCO_3^- nor in Na^+/HCO_3^- free medium, we checked by viability tests with MTT which are the vital consequences for the cells in those conditions. We observed a time-dependent decrease in cell viability in HMC-$1^{560, 816}$ cells (Fig. 3B, D) but not in HMC-1^{560} cells (Fig.

3A, C) incubated in saline solution without HCO_3^- and $NaHCO_3$. HMC-1^{560} cell viability was unaffected after 30 minutes of incubation in the absence of HCO_3^- or when Na^+ and HCO_3^- were absent (Fig. 3A). Similar results were obtained after one hour of incubation (Fig. 3C). In contrast, after 30 minutes of incubation without HCO_3^- HMC-$1^{560, 816}$ cell viability was decreased about 5% (Fig. 3B), and in the absence of HCO_3^- and Na^+ the decrease was about 18%. After one hour of incubation (Fig. 3D) in HCO_3^- free medium cell viability was significantly decreased by 22% and about 21% in Na^+/HCO_3^- free medium. These results agree with our observations that HMC-1^{560} cells might be less sensitive to the extracellular conditions. However we can conclude that both extracellular HCO_3^- and a functional HCO_3^-/CO_2 buffer system are required for HMC-$1^{560, 816}$ cell survival. After these results, next we studied if the inhibition of anion transport does also modify HMC-1 viability. Stilbene disulphonate derivatives such as 4,4'- diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt (DIDS) and the amino reactive agent 4-acetamido-4'-isothiocyanato-stilbene-2,2'-disulfonic acid (SITS) and diphenylamine-2-carboxylate derivatives such as 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) are shown to inhibit anion transport across the cell membrane. Therefore we performed viability tests after prolonged incubation with both sublines with different concentrations of these inhibitors. As Fig. 4 shows, incubation of HMC-1^{560} and HMC-$1^{560, 816}$ cells for 24 hours with different doses of DIDS (Figs. 4B, E) or NPPB (Figs. 4A, D) decreased cell viability in concentrations up to 200µM. The incubation with SITS had no effect in any of the sublines (Figs. 5C, F). Our results indicate that anion exchanger modulation affects HMC-1 cell viability. Hereupon we proved if the effects evoked by these inhibitors were directly linked to an intracellular pH decrease.

Since HMC-$1^{560, 816}$ cells appeared to be more sensitive to the presence or absence of extracellular HCO_3^-, further experiments were performed with these cells. Cells were firstly incubated for 5 minutes with NPPB (200µM) in physiologic saline solution and afterwards the extracellular medium was replaced with a HCO_3^- free one. As Fig 5A shows pH_i

significantly decreased stronger in NPPB pre-treated cells than in control cells. No differences were observed in cells incubated with DIDS (200µM, Fig. 5B). Pre-incubation with neither NPPB nor DIDS affected pH_i in saline solution containing HCO_3^- (data not shown). As we have observed a Na^+ dependent pH_i drop in HCO_3^- free solution and some anion transporters are inhibited by amiloride, HMC-$1^{560,\ 816}$ cells were also incubated with its derivate EIPA. Cells pre-incubated with EIPA (5µM, Fig. 5C) demonstrated a stronger pH_i fall than non pre-incubated cells. This observation matched our previous results in Figs. 1 and 2, HCO_3^- transport in these cells is Na^+ dependent and the absence of this cation affects pH_i steady-state in HCO_3^- free saline solution. Several anion exchangers are reported to be directly linked with carbonic anhydrase (CA) II and IV. Therefore HMC-$1^{560,\ 816}$ cells were pre-incubated with acetazolamide (500µM, AAA) a known CA inhibitor. Fig. 5D shows that AAA-treated cells demonstrated less pH_i falling than control cells in HCO_3^- free saline solution. As CAs catalyze the reaction of $H_2O + CO_2 <-> HCO_3^- + H^+$ this result indicates that an increase in intracellular HCO_3^- can compensate the pH_i fall evoked by the absence of extracellular HCO_3^- .

Furthermore the Na^+ dependence of pH_i regulation in HMC-1 cells could not be clearly identified as a regulating mechanism by itself which influence a HCO_3^- transporter or as the consequence of a direct inhibition of a Na^+ dependent HCO_3^- mechanism. Therefore we checked the expression of NHE1 in the presence and absence of HCO_3^- in both sublines. Fig. 6A shows that NHE1 expression in HMC-1^{560} cells is not affected after 10 minutes of incubation in Na^+/HCO_3^- free medium. In contrast, as Fig. 6B demonstrates, NHE1 expression is strongly increased in HMC-$1^{560,\ 816}$ cells bathed in Na^+/HCO_3^- free saline solution compared with those in Umbreit. The very same blots were utilized for reblotting with ß-tubulin which was used as loading control and the corresponding ratio values were calculated. The ratio values of NHE1/ ß-tubulin expression in HMC-1^{560} cells with and without Na^+/HCO_3^- were almost the same (Fig. 6C). In HMC-$1^{560,\ 816}$ cells ratio values were higher in cells incubated without Na^+/HCO_3^- which clearly confirms the involvement of

NHE1 in the pH_i decrease of these cells. Once more we can observe the different sensibility of both sublines; cells with two mutations seem to be more sensible to the replacement of external Na^+ andHCO_3^-.

DISCUSSION

Human Mast Cells (HMC-1) are not able to stabilize their basal pH_i when extracellular HCO_3^- is absent. Intracellular pH immediately decreases when cells are placed in medium without HCO_3^-. It seems that the CO_2/HCO_3^- buffer system is the main pH_i stabilizing mechanism in HMC-1 cells. This matches with the observation that pH_i falling stops in HMC-$1^{560, 816}$ cells when extracellular pH is increased. When HCO_3^- and Na^+ are changed in the medium, the pH_i decrease is even stronger. The same effect can be observed if one of the main Na^+ influx mechanisms, the Na^+/H^+ exchanger, is inhibited by EIPA. Furthermore, the addition of $NaHCO_3$ but not $KHCO_3$ stabilized the pH_i decline. The expression and activation of NHE1 could be confirmed HMC-$1^{560, 816}$ cells in the absence of extracellular Na^+ and HCO_3^-. In conclusion HCO_3^- regulation in HMC-1 cells is strongly Na^+ dependent and NPPB sensitive as pH_i drop was even higher when cells were pre-treated with the inhibitor. We can not demonstrate its direct inhibition by DIDS, but its sensitivity against the amiloride derivate EIPA. This effect can be caused by a direct inhibition of the HCO_3^- mechanism which is reported in physiologic studies of NBCn1 or to the reduced Na^+ influx which indirectly affects the Na^+-dependent transporter (Choi et al., 2000; Fliegel, 2005; Slepkov et al., 2007). In order to examine which HCO_3^- transporter is responsible for the destabilisation of pH_i steady-state of HMC-1 cells in HCO_3^- free condition we could exclude the Na^+ independent anion exchangers (AEs) and concentrate in those which require Na^+ for their function. Cells may also express further transporters which are not activated by our study conditions. NBCe1 is an electrogenic transporter and predominantly expressed in the kidney proximal tube where it plays an important role in HCO_3^- reabsorption. Furthermore the transporter can be found in pancreatic duct cells where it secretes HCO_3^- (Soleimani and Burnham,

2001). This transporter is well studied and fulfils very special functions what are not related to mast cell function or HMC-1 distribution. We also excluded the NBCe2 transporter on the one hand for its electrogenicity and on the other for its strong inhibition by DIDS. NBCn1 and NDCBE are described to regulate pH_i and are widely distributed in several tissues. The former was found in human retina, spinal cord, muscle, thymus, and other tissues and to be overexpressed in cancer cells (Choi et al., 2000; Izumi et al., 2003). Functional studies in oocytes indicate an electroneutral Na^+/HCO_3^- transport independent of Cl^-. Furthermore NBCn1 is described as DIDS insensitive but sensitive to amiloride. Both descriptions match our results in HMC-1 cells, however a posterior study with a rat NBCn1 homologue demonstrated amiloride resistance and a partial sensibility to DIDS (Choi et al., 2000; Pushkin et al., 1999a; Pushkin et al., 1999b). The other possible Na^+ dependent HCO_3^- transporter that could regulate pH_i and HCO_3^- transfer in HMC-1 cells is the NDCBE transporter. Besides it was found in testis, ovary and brain this exchanger is expressed in the bone marrow where mast cells have their origin (Kirshenbaum et al., 1991). It is a matter of controversy if the electroneutral transporter requires Cl^- for its function, since Cl^- depletion did not affect Na^+/HCO_3^- cotransport in one study but in another report NDCBE expressing oocytes demonstrated a Na^+ dependent Cl^-/HCO_3^- exchanging mechanism (Amlal et al., 1999; Grichtchenko et al., 2001). HMC-1 cells have strong voltage dependent Cl^- currents (CIC-3 and CIC-5) as well as Ca^{2+} activated Cl^- currents (Duffy et al., 2003; Duffy et al., 2001). Therefore the HCO_3^- transporter described in this study might regulate HCO_3^- exchange dependant to Cl^- to facilitate the extrusion of this anion.

The results are contrary to reports in rat mast cells, where the main HCO_3^- mechanism is a Na^+ independent Cl^-/HCO_3^- exchanger. It is to bear in mind that HCO_3^- transporters are function and tissue specifically expressed. Different cell types posse distinct transporters and even cells of the same tissue express according to their role specific exchangers. As HMC-1 cells were obtained from a patient with mast cells leukaemia their function might be distinct than that of normal mast cells obtained from

rats. Another consideration is that mast cell transporter expression probably depends on the tissue of which cells were extracted. In this study we observe differences in the sensibility of their HCO_3^- transporters between HMC-1^{560} and HMC-$1^{560, 816}$. This is not unexpected, since mast cells with mutations at 560 and 816 appear in different forms of mastocytosis. The CO_2/HCO_3^- buffer system of every cell type is adapted to their specialized function and distribution. Mast cells with 560 mutations appear in patients with cutaneous mastocytosis, and accordingly they are more dispersed in the skin, whereas 816-cells appear in cases of systemic mastocytosis and are therefore distributed in much more tissues. The higher sensibility of HMC-$1^{560, 816}$ cells compared with HMC-1^{560} cells could be due to their wider tissue distribution.

The early phase of apoptosis is triggered by cellular acidosis which leads to endonuclease activation and DNA fragmentation. Therefore in tumour cells pH regulators like NHEs and NBCs are thought to be up-regulated. In this consideration it is interesting that HMC-1 cells are sensible to the absence or presence of HCO_3^-. In the absence of extracellular HCO_3^-, pH_i decreases and cells are not able to stabilize steady-state pH by another mechanism. Hereupon cell viability is reduced as when anion exchangers are inhibited by DIDS or NPPB. Our results give new clues for a possible drug targeting in mastocytosis since intracellular pH is crucial for biological cell functions. Modulation of pH_i as side effects induced by pharmaceuticals and metabolites should also be considered in patients with mastocytosis. It is therefore important that just an alkalinisation can provoke HMC-1 cell exocytosis.

This study is the first report of the existence of a Na^+-dependent HCO_3^- regulating mechanism in human mast cells. External HCO_3^- is necessary for cellular steady-state pH in HMC-1 cells. Regulation might take place through the NCBn1 or the NDCBE transporter. In every case the transporter shows different sensibility in HMC-1^{560} and HMC-$1^{560, 816}$ cells. A possible overexpression of the exchanger which is described in this study may contribute to the malignant phenotype of these cells. External HCO_3^- as well as functioning of HCO_3^- exchange is important for HMC-1 cell viability.

FIGURES

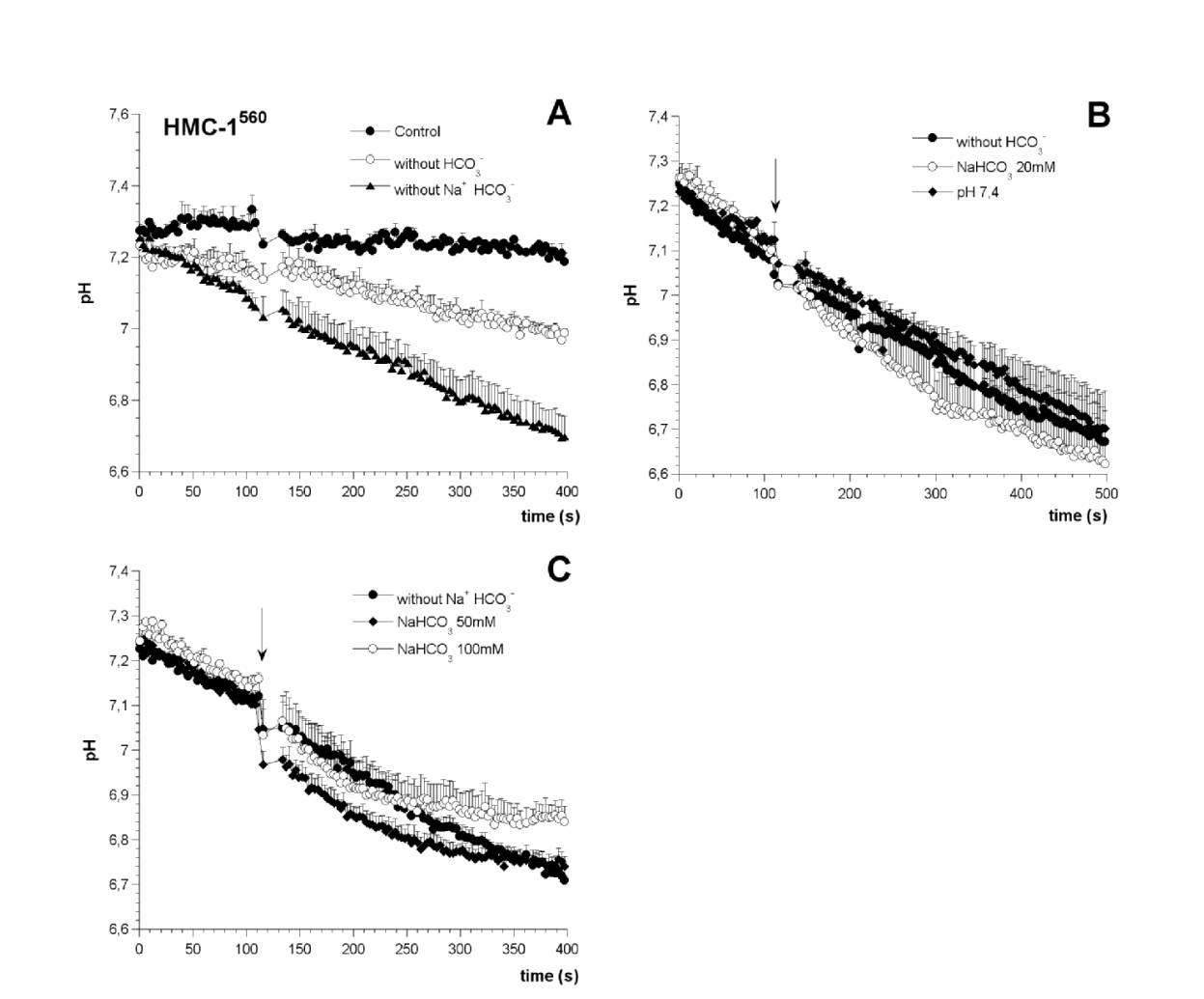

Figure 1: Effect of HCO_3^- and Na^+/HCO_3^- free saline solution on pH_i of HMC-1^{560} cells Intracellular pH profile: **(A)** HCO_3^- free and Na^+/HCO_3^- free saline solutions (extracellular pH=7.2 was constant during the whole experiment). **(B)** extracellular HCO_3^- free buffer was changed to $NaHCO_3$ (20mM) containing medium or to HCO_3^- free buffer (pH=7.4). **(C)** extracellular medium was changed to $NaHCO_3$ (50mM or 100mM) containing buffer. The arrows indicate the point of buffer exchange. Mean+/-SEM of three experiments.

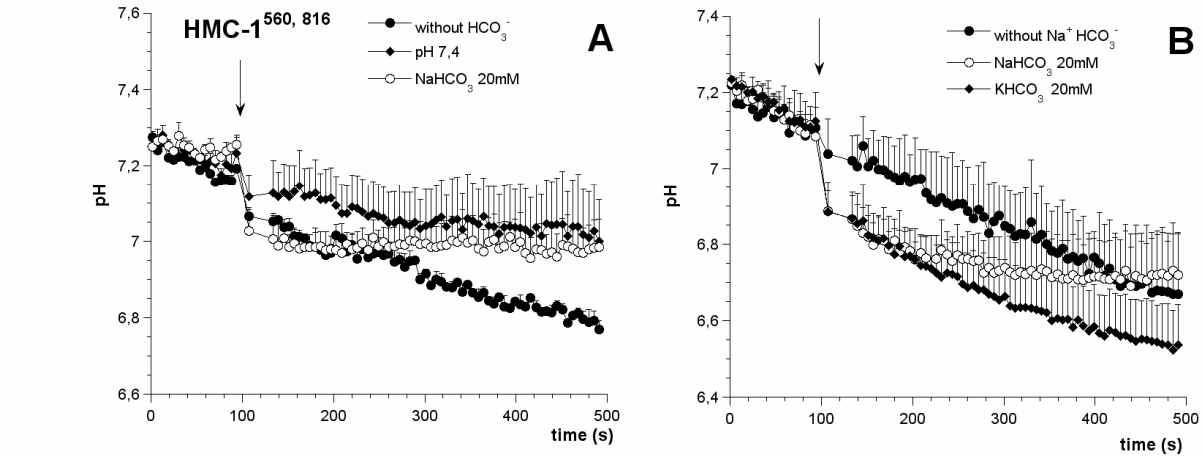

Figure 2: Effect of HCO_3^- and Na^+/HCO_3^- free saline solution on pH_i of HMC-1$^{560, 816}$ cells Intracellular pH profile: **(A)** HCO_3^- free extracellular medium (pH=7.2) was changed to $NaHCO_3$ (20mM) containing medium or to HCO_3^- free buffer (pH=7.4). **(B)** Na^+/HCO_3^- free saline solution (pH=7.2) was changed to $NaHCO_3$ (20mM) or $KHCO_3$ (20mM) containing saline solution (pH=7.2). The arrows indicate the point of buffer exchange. Mean+/-SEM of three experiments.

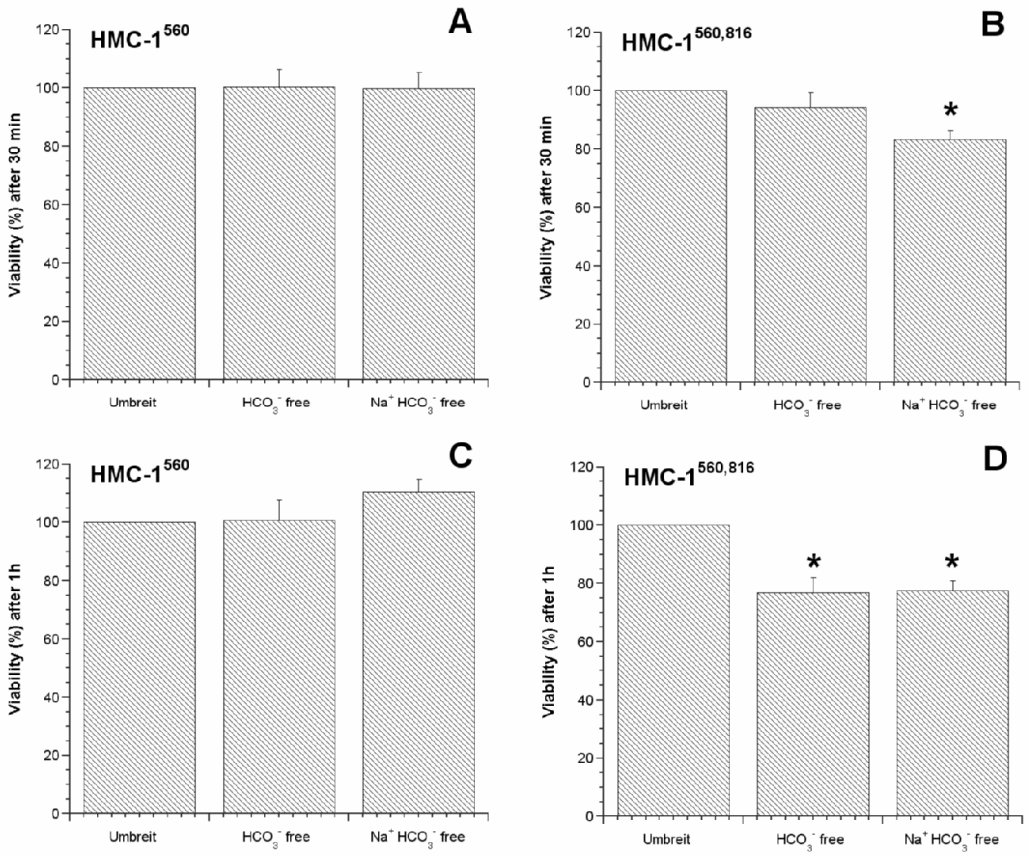

Figure 3: HMC-1^{560} and HMC-1$^{560, 816}$ cell viability after 30 minutes and one hour incubation in different extracellular mediums. Cells were incubated in Umbreit, HCO_3^- free and Na^+/HCO_3^- free saline solution (pH=7.2) for 30 minutes **(A, B)** and one hour **(C, D)** at 37 ºC in a water bath. Cell viability was checked by MTT test. Control cells in Umbreit were used as 100 % viability in each experiment. Mean ± SEM of three experiments.

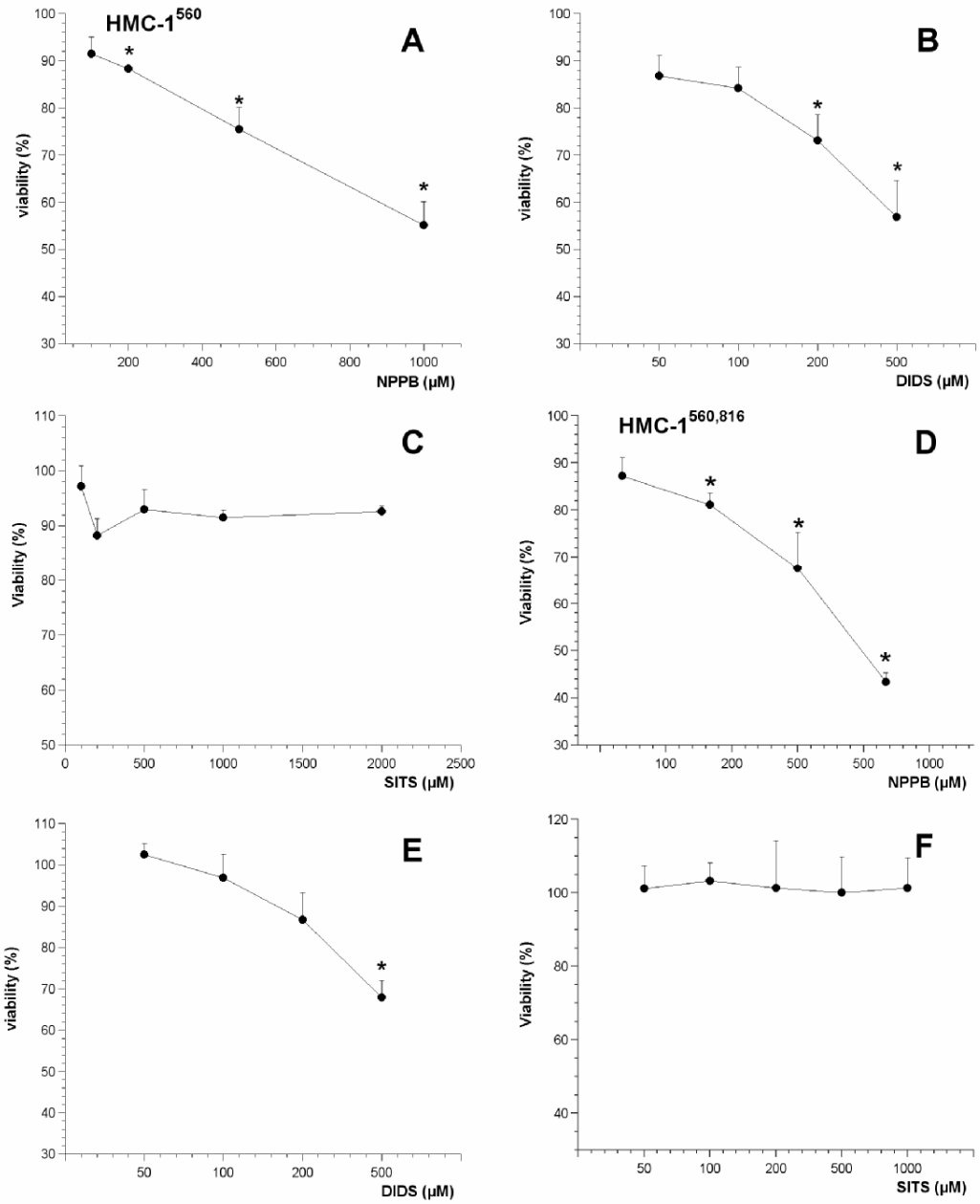

Figure 4: HMC-1^{560} and HMC-1$^{560, 816}$ cell viability after 24 hours of incubation with anion exchanger inhibitors. Different concentrations of DIDS, NPPB and SITS were added in culture medium and cells were incubated for 24 hours at 37 ºC, 5% CO_2. Cell viability was checked by MTT test. Control cells with non-inhibitor treatment, were used as 100 % viability in each experiment. Mean ± SEM of three experiments.

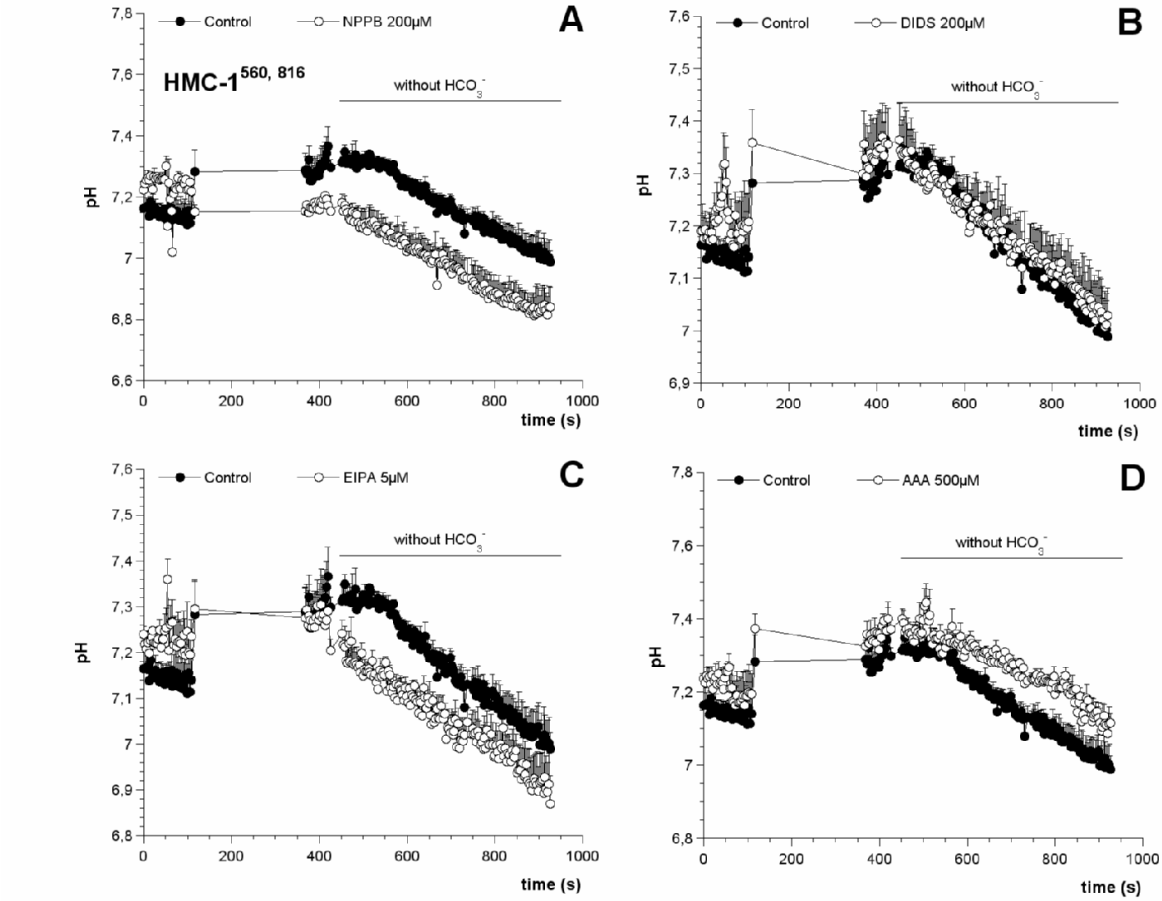

Figure 5: Effect of anion exchanger modulation on acidifying pH_i in HCO_3^- free conditions in HMC-1$^{560, 816}$ cells. Intracellular pH profile: cells were treated during 5 minutes in Umbreit with **(A):** NPPB (200µM), **(B):** DIDS (200µM), **(C):** EIPA (5µM) and **(D):** Acetazolamide (AAA, 500µM). Afterwards extracellular medium was changed to HCO_3^- free buffer. Mean+/-SEM of three experiments.

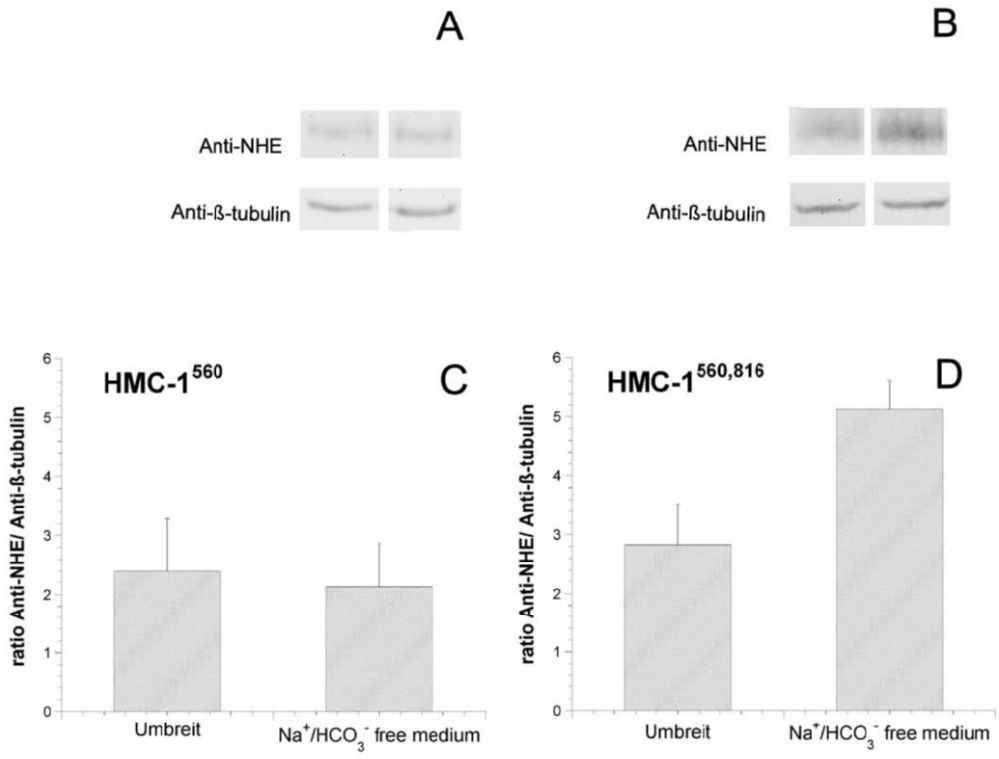

Figure 6: Effect of Na^+/HCO_3^- free saline solution on NHE expression of HMC-1^{560} and HMC-$1^{560, 816}$ cells. NHE expression of HMC-1^{560} **(A, C)** and HMC-$1^{560, 816}$ cell **(B, D)** after 10 minutes of incubation in Umbreit or Na^+/HCO_3^- free saline solution. Mean+/-SEM of three experiments.

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II.B: NH_4Cl Induced Alkalinisation and Exocytosis of HMC-1 Human Mast Cells are Regulated by NHE1 and a Na^+/HCO_3^- Exchanger

NH_4Cl induced alkalinisation and exocytosis of Human Mast Cells HMC-1 are regulated by a Na^+/HCO_3^- exchanger and NHE1

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ABSTRACT

The Human Mast Cell lines (HMC-1^{560} and HMC-$1^{560,816}$) were used to study the role of external Na^+ and HCO_3^- on NH_4Cl induced alkalinisation and the subsequent histamine release. HMC-1^{560} and HMC-$1^{560,816}$ cells carry activating mutations in the proto-oncogene c-kit that cause autophosphorylation and permanent KIT TyrK activation. These mutations lead to a Gly-560 -> Val exchange in both sublines and to an Asp-816 -> Val amino acid change in HMC-$1^{560, 816}$ cells.

NH_4Cl induced alkalinisation was significantly decreased in the absence of extracellular HCO_3^-. The decrease was even stronger when besides HCO_3^- as well external Na^+ was substituted. This behaviour was more notable in HMC-1^{560} cells. When Na^+ and HCO_3^- were absent histamine release was almost abolished in HMC-1^{560} cells while in HMC-$1^{560,816}$ cells exocytosis was only diminished in the same conditions. DIDS, NPPB and tamoxifen were employed to inhibit anion exchange activity in physiologic saline solution but no effects were observed either on alkalinisation or exocytosis induced by NH_4Cl. Further results show that the absence of extracellular Cl^- did not alter NH_4^+ induced alkalinisation or histamine release.

In addition Na^+/H^+ exchanger (NHE1) inhibition inhibits alkalinisation while histamine release is not affected. This study demonstrates that NH_4Cl induced alkalinisation and exocytosis are strongly dependent on the presence of extracellular Na^+ and HCO_3^- and the functioning of NHE1.

Abbreviations: HMC-1: Human Mast Cell Line; NH_4Cl: ammonium chloride; pH_i: intracellular pH

INTRODUCTION

HMC-1^{560} and HMC-$1^{560, 816}$ cells are mast cell lines obtained from a patient with mast cells leukaemia. Both express mutations in their TyrK receptor KIT (Sundstrom et al., 2003), that constitutively activate the receptor without binding to its ligand stem cells factor (SCF). These mutations appear in the proto-oncogene c-kit, which encodes the KIT protein. These lead to an amino acid exchange in the protein on the one hand in Gly-560->Val (HMC-1^{560}), and on the other Asp-816->Val (HMC-$1^{560, 816}$). The change in the position 560 results in the permanent ligand independent activation of both sublines. The mutation is common in various human malignant diseases, including gastrointestinal stromal tumour and cutaneous mastocytosis (Shah et al., 2006). A second c-kit mutation, which express HMC-$1^{560, 816}$ cells leads to Asp-816->Val change and modifies the conformation of the intracellular TyrK and modifies thereby their drug responses (Akin et al., 2003; Ma et al., 2002; Yavuz et al., 2002). This mutation could be identified in more than 80% of systemic mastocytosis cases (Akin and Metcalfe, 2004; Garcia-Montero et al., 2006).

Intracellular pH (pH_i) plays an important role in mast cell exocytosis and activation. The Na^+/ H^+ exchanger 1 (NHE1) is ubiquitously expressed in the integral plasma membrane of almost all cell types and can be inhibited by amiloride and its derivates. Intracellular acidification activates the ion transporter and changes sodium from extracellular against intracellular protons. NHE1 consists of 12 transmembrane domains and a hydrophilic tail, which are target for protein kinases and the binding site for regulatory proteins. ERK via MAPK, $p90^{rsk}$, NIK and CAMKII directly phosphorylate and activate NHE1, PKC and PKD induce higher NHE activity but not by direct phosphorylation. The transporter alters its pH dependence in presence of Ca^{2+} modulators like calmodulin and CHP, which activate the exchanger and tescalcin, which reduces its activity (De Vito, 2006; Fliegel, 2005; Slepkov et al., 2007). In rat mast cells NHE1 is suggested to be an important sodium influx mechanism as well as intracellular pH (Botana et al., 1992; Cabado et al., 1993). After

an artificial acidification rat mast cell recovery rate is two-fold smaller in amiloride treated cells. Whereas Ca^{2+} suppression does no affect NHE activity, thapsigargin (Ca^{2+} ATPase inhibitor) and PMA (PKC stimulation) are able to activate this exchanger. Increased cAMP activity does not modify PKC effect on recovery but blocked that induced by thapsigargin (Alfonso et al., 1998). The modulation of different signal transduction pathways on NH_4Cl induced degranulation have been studied since it has been demonstrated that cytosolic alkalinisation is a sufficient signal for rat mast cell exocytosis (Alfonso et al., 2000; Alfonso et al., 2005).

In HMC-1^{560} and HMC-$1^{560, 816}$ cells NH_4Cl addition dose-dependently increases pH_i without modifying basal cytosolic Ca^{2+} concentrations. This alkalinisation does induce degranulation without any cytosolic Ca^{2+} increase. PKC activation increases NH_4Cl induced alkalinisation and leads on to further histamine release. Similar data is obtained after increasing cAMP levels by forskolin and dibutyryl cAMP. Those results suggest a NHE involvement in alkalinisation induced exocytosis of HMC-1^{560} and HMC-$1^{560, 816}$ cells (Lober et al., 2008a; Lober et al., 2008b; Pernas-Sueiras et al., 2005; Pernas-Sueiras et al., 2006a; Pernas-Sueiras et al., 2006b).

Another important mechanism in pH_i regulation, which seems to be implicated in mast cell exocytosis, is the CO_2/HCO_3^- buffer system. Different HCO_3^- transporters are function-specifically expressed and act in a tissue dependent specialized mode. They can be divided in three major groups: Na^+ independent Cl^-/HCO_3^- exchanger (AE), Na^+ HCO_3^- cotransporters (NBC) and Na^+ driven Cl^-/HCO_3^- exchangers (NCBE, BTR1). AEs are mainly activated during intracellular alkalinisation and transport HCO_3^- out of the cell in exchange to extracellular Cl^-. Binding to carbonic anhydrase II and IV stimulates their transporter activity. Na^+/HCO_3^- cotransporter alkalinize intracellular pH by triggering HCO_3^- influx, in a similar fashion as Na^+ driven Cl^-/HCO_3^- exchangers in a Cl^- dependent manner. Almost all HCO_3^- transporters can be inhibited by stilbene derivates like DIDS and some of them by amiloride analogists (Romero et al., 2004).

In rat peritoneal mast cells a Na^+ independent pH_i fall was observed when the external buffer contained HCO_3^-. Removal of extracellular Cl^- reversed Cl^-/HCO_3^- exchange and induced a Na^+ independent alkaline load. Alkalinisation and its pH_i recovery could be inhibited by DIDS and were HCO_3^- dependent. The same study demonstrated that histamine release was reduced in presence of HCO_3^- due to changes in ion transport and not to effects on pH (Jensen et al., 1998). It has been shown that rat mast cells undergo alkalinisation in HCO_3^--free extracellular medium after $NaHCO_3$ addition. The effect was minor adding $KHCO_3$. Increase was inhibited by amiloride and DIDS suggesting that alkalinisation is controlled by NHE and AE regulating mechanisms in these cells (Vilarino et al., 1998). In serosal mast cells HCO_3^- significantly reduced histamine release induced by PMA and A23187/ PMA but increased histamine release induced by thapsigargin. The effect of HCO_3^- depended on the stimulus and was not related to Cl^- presence (Vilarino et al., 1999).

The aim of this work was to find out which pH mechanisms regulate alkalinisation and followed histamine release in HMC-1^{560} and HMC-$1^{560, 816}$ cells. The knowledge of pH regulation and its role in degranulation in these cell lines is important since cells with those mutations are expressed by patients with different forms of mastocytosis and their exocytosis could be induced with just an intracellular alkalinisation.

METHODS

Chemicals

NH_4Cl was from Panreac (Barcelona, Spain); 2,7-bis (carboxyethyl)-5(6)carboxy-fluorescein-acetoxymethylester (BCECF AM) was from Molecular Probes (Leiden, The Netherlands); DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt), NPPB (5-nitro-2-[3-phenylpropylamino] benzoic acid), EIPA, Acetazolamide, Tamoxifen, MTT (3-[4,5-dimethyldiazol-2-yl]-2,5-diphenyltetrazolium bromide) were from Sigma-Aldrich (Madrid, Spain).

Cell cultures

HMC-1 cells were kindly provided by Dr. J. Butterfield (Mayo Clinic, Rochester, MN) and maintained in Iscove´s modified Dulbecco´s medium (IMDM) supplemented with 10 % fetal bovine serum (FBS), 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were expanded weekly and not more than 40 passages.

Cell preparation

Cells were centrifuged (1500 r.p.m., 5 minutes, 4 ºC) and washed twice with saline solution (1000 r.p.m., 5 minutes, 4 ºC). The composition of this solution (Umbreit) was (mM): Na^+ 142.3; K^+ 5.94; Ca^{2+} 1; Mg^{2+} 1.2; Cl^- 126.2; HCO_3^- 22.85; HPO_4^{2-} 1.2, SO_4^{2-} 1.2; glucose 1 g/l. The composition of the HCO_3^- free solution was (mM): Na^+ 139.19; K^+ 5.94; Mg^{2+} 1.2; Cl^- 124.94; HPO_4^{2-} 7.7; $H_2PO_4^-$ 7.7; SO_4^{2-} 1.2; glucose 1 g/l. The composition of the Na^+/HCO_3^-- free solution was (mM): K^+ 5.94; Ca^{2+} 1; Mg^{2+} 1.2; choline 143; Cl^- 149.94; SO_4^{2-} 1.2; Tris-base 8; Tris-HCl 41.95; glucose 1 g/l.

For pH measurements cells were treated and washed in Umbreit solution plus 0.1 % bovine serum albumin (BSA). The incubation medium was equilibrated with CO_2 prior to use. During the experiments, pH (7.20) was maintained constant by bubbling CO_2. Experiments were carried out at least three times, by duplicate.

Measurement of intracellular pH

HMC-1 cells were loaded BCECF AM (0.05 µM) in a bath at 37 ºC, for 10 minutes. After this time, loaded cells were washed with saline solution (1000 r.p.m., 10 minutes, 4 ºC). Cells were attached to glass coverslips treated with poly-L-lysine, and these were inserted into a thermostated chamber (Life Sciences Resources, U.K.). Cells were viewed using a Nikon Diaphot 200 microscope equipped with epifluorescence optics (Nikon 40x– immersion UV– Fluor objective). Addition of drugs was made by aspiration and addition of fresh bathing solution to the chamber. Intracellular pH was obtained from the images collected by

fluorescence equipment (Life Sciences Resources, U.K.). The light source was a 175 W xenon lamp, and the used wavelengths were selected with filters. For BCECF AM the excitation was performed at 440 and 490 nm, with 530 nm for emission. The calibration fluorescence values *vs.* pH as per Thomas *et al*. (Thomas et al., 1979). In brief, a calibration curve was obtained with four known values of pH, measuring the fluorescence ratio obtained in the presence of nigericin, in a K^+ solution, for each pH value.

Statistical analysis.
Results were analyzed using the Student's t-test for unpaired data. A probability level of 0,05 or smaller was used for statistical significance. Results were expressed as the mean ± SEM.

RESULTS

In this work we investigated how NH_4Cl induced alkalinisation is regulated in HMC-1^{560} and HMC-$1^{560,816}$ cells and which mechanisms affect the subsequent histamine release.
It has been shown previously that alkalinisation by itself induces histamine release in both sublines without modulating cytosolic Ca^{2+} levels (Lober et al., 2008b; Pernas-Sueiras et al., 2005). It has been observed that in both sublines pH_i is regulated by Na^+ and HCO_3^- dependent mechanisms (unpublished results). Therefore HMC-1^{560} cells were treated with NH_4Cl in different saline solution without HCO_3^- or without Na^+/HCO_3^- to observe intracellular pH. As previously reported pH_i dramatically decreased in these cells in the absence of extracellular HCO_3^- whereas the external pH was maintained constant during the whole experiment. Fig. 1A shows pH_i augmented from 7.10 to 7.14 (ΔpH_i =+0.04) in this medium after addition of NH_4Cl (20mM). The pH_i increase in Na^+/HCO_3^- free conditions was about +0.03 units and not significant (Fig. 1B). Comparative experiments were performed with higher NH_4Cl concentrations to demonstrate the differences of the effect of the compound in physiologic saline solution and in HCO_3^- or Na^+/HCO_3^- free solution. As Fig. 1C shows the addition of NH_4Cl (50mM) to saline

solution containing Na^+ and HCO_3^- increased pH_i about 0.24 units at the addition point while in HCO_3^- free medium, pH_i only rose about 0.14 units and just about 0.1 units in saline solution without Na^+/HCO_3^-. Afterwards in every case pH_i slowly recovered towards base levels. Similar results were obtained adding NH_4Cl (100mM, Fig. 1D). In physiologic saline solution pH_i rises up from 7.24 to 7.58 (ΔpH_i =0.33) whereas in the absence of HCO_3^- the increase was about 0.16 and about 0.24 units without Na^+/HCO_3^-. In the case of adding 50mM or 100mM of NH_4Cl, while in HCO_3^- free medium pH_i recovery reached base levels around 7.2 and stopped its decrease, in Na^+/HCO_3^- free medium the fall continued after recovery. Our results indicate that the alkalinisation provoked by NH_4Cl in HMC-1^{560} is strongly dependent on extracellular Na^+ and HCO_3^- and that Na^+ is necessary to stabilize pH_i after recovery from an alkaline load. The same experiments were performed with HMC-$1^{560,816}$ cells. Fig 2A shows that NH_4Cl (20mM) provoked a pH_i increase from 7.07 to 7.25 (ΔpH_i =0.18) in the absence of external HCO_3^-. After alkalinisation pH_i recovered and stabilized around 7.00. The pH_i of non-alkalinized cells continued its fall. When NH_4Cl (20mM) was added to cells in Na^+/HCO_3^- free medium the pH_i rose about 0.07 values and decreased afterwards (Fig.2B). Alkalinized cells did not stop pH_i decrease neither reach that low values as non-alkalinized cells. HMC-$1^{560,816}$ cells were more sensitive to NH_4Cl as their pH_i significantly augmented with NH_4Cl (20mM) in all extracellular solutions. In Fig. 2C previous results are compared with those obtained in physiologic saline solution and it is evident that Na^+ and HCO_3^- are necessary to induce alkalinisation in these cells. In addition as it could be observed in HMC-1^{560} cells, Fig. 2 C clearly shows that pH_i only stabilizes after recovery from a basic load when extracellular Na^+ is present.

We next studied the consequences of this behaviour for exocytosis since NH_4Cl dose-dependently induces histamine release, this release being matched with the pH_i increase induced by each NH_4Cl dose (Pernas-Sueiras et al., 2005). Histamine release induced by NH_4Cl was reduced in HMC-1^{560} cells (Fig.3A) in the absence of HCO_3^- but it did not alter mediator release of HMC-$1^{560,816}$ cells (Fig. 3B). These results matched

with the previous ones: the same NH_4Cl (20mM) concentration induced less pH_i increase in HMC-1^{560} cells (Fig 1A) than in HMC-$1^{560,816}$ cells (Fig. 2A). In Fig 3C, histamine release was almost completely abolished in HMC-1^{560} cells treated with low NH_4Cl concentrations without external Na^+/HCO_3^-. Even when the differences were not significant HMC-$1^{560,816}$ released also less histamine in these conditions (Fig. 3D). Our results indicate that Na^+ and HCO_3^- dependent exchanger are involved in NH_4Cl induced degranulation and that the amount of released histamine correlates with pH_i increase evoked by the compound.

Stilbene disulphonate derivatives such as 4,4'- diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt (DIDS) and diphenylamine-2-carboxylate derivatives such as 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) inhibit anion transport. According to our results we checked the effect of these substances on alkalinisation induced histamine release. As Fig. 4 shows pre-incubation of HMC-1^{560} and HMC-$1^{560,816}$ cells with DIDS (200µM, Figs. 4A, E) or NPPB (200µM, Figs. 4B, F) did not alter exocytosis. Pre-incubation with neither NPPB nor DIDS affected NH_4Cl induced alkalinisation in physiologic saline solution containing Na^+ and HCO_3^- (data not shown). A great part of HCO_3^- transport mechanisms function in exchange with Cl^-. Since HMC-1 cells consist of strong voltage dependent Cl^- currents (CIC-3 and CIC-5) which could be inhibited by tamoxifen we also tested if this inhibitor modulates NH_4Cl induced histamine release (Duffy et al., 2003; Duffy et al., 2001). Tamoxifen did not affect exocytosis of HMC-1^{560} (Fig. 4C) nor HMC-$1^{560,816}$ cells (Fig. 4G). Some anion exchangers work directly with carbonic anhydrase (CA) II and IV. Therefore both HMC-1 sublines were pre-incubated with acetazolamide (500µM, Fig. 4D, H) a known CA inhibitor. Histamine release was not reduced after acetazolamide treatment. The exchanger involved in alkalinisation-induced histamine release might be insensitive to the utilized inhibitors or the experimental conditions did not allow detecting an effect. As mentioned above many anion mechanisms such as AEs and NDCBE transport HCO_3^- in exchange to Cl^- through the cell membrane. Therefore we tested if NH_4Cl induced alkalinisation depends on extracellular Cl^-. HMC-1^{560} (Fig. 5A) and HMC-

$1^{560,816}$ (Fig 5C) were incubated in saline solution without Cl^- and alkalinisation was induced by $(NH_4)_2SO_4$ (20mM) or NH_4Cl (20mM). $(NH_4)_2SO_4$ and NH_4Cl alkalinized both cell sublines with the same intensity. We also checked the Cl^- dependence of NH_4Cl induced histamine release. HMC-1^{560} (Fig. 5B) and HMC-$1^{560,816}$ (Fig. 5D) were stimulated by different concentrations of $(NH_4)_2SO_4$ in the absence and presence of Cl^- but no statistical differences could be observed in non of the sublines. NH_4Cl induced alkalinisation and the subsequent mast cell degranulation are clearly Cl^- independent processes.

As we have observed a Na^+-dependent pH_i increase after alkalinisation the role of the Na^+/H^+ exchanger (NHE) was tested in this correlation. NHE of HMC-$1^{560,816}$ cells was inhibited by the amiloride derivate EIPA (5µM) and pH_i was monitored. As Fig. 6A shows alkalinisation could be significantly inhibited after 5 minute pre-treatment with the inhibitor. Hereupon the effect on histamine release was checked, but as Fig. 6B demonstrates EIPA pre-treatment did not reduce histamine release induced by NH_4Cl. This was not surprising since it has been shown that just a shift in pH_i could activate exocytosis in HMC-1 cells (Pernas-Sueiras et al., 2005).

DISCUSSION

NH_4Cl induces a rapid and dose-dependent alkalinisation in HMC-1^{560} and HMC-$1^{560,816}$ cells. This process leads to exocytosis independently of the presence of extracellular Ca^{2+} and without modulating cytosolic Ca^{2+} levels (Lober et al., 2008b; Pernas-Sueiras et al., 2005).

In this work we studied which pH regulating mechanisms participate in this process and how far they are involved in HMC-1 degranulation. At first we checked the CO_2/HCO_3^- buffer system as we have seen that these cells express a Na^+-dependent HCO_3^- transporter to stabilize steady-state pH_i (unpublished results). Our results demonstrate that alkalinisation is significantly diminished in the absence of HCO_3^-. The effect is more notable when both HCO_3^- and Na^+ are absent, which advices to the activity of a Na^+ dependent HCO_3^- transporter. In these

conditions alkalinisation induced histamine release is reduced in HMC-$1^{560,816}$ cells and almost abolished in HMC-1^{560} cells. These results clearly indicate the importance of a functional Na^+/HCO_3^- mechanism for alkalinisation-induced histamine release. Unfortunately anion exchange inhibitors like NPPB and DIDS do not impair alkalinisation or exocytosis. This can be due to experimental conditions or to the possible resistance of the participating transporter against these inhibitors as it is reported in the case of NBCn1 (Choi et al., 2000; Pushkin et al., 1999a; Pushkin et al., 1999b). The importance of extracellular HCO_3^- for mast cell exocytosis has been shown in several studies (Jensen et al., 1998; Lee et al., 1992; Vilarino et al., 1999). In rat peritoneal mast cells steady-state pH was lower in HCO_3^- containing extracellular medium and antigen- or compound 48/80-induced histamine release was reduced. It was suggested that the reduction in exocytosis was not due to its effect on pH_i but rather on other ion changes (Jensen et al., 1998). Later on it could be demonstrated in serosal rat mast cells that the presence of HCO_3^- affected Ca^{2+} entry and degranulation depending on the used stimulus (Vilarino et al., 1999). In RBL-2H3 rat basophilic leukaemia cells Ca^{2+} entry and degranulation induced by ionomycin were reduced following HCO_3^- removal due to a subsequent acidification and the inactivation of a HCO_3^- dependent mechanism (Lee et al., 1992). In HMC-1 cells the effect of HCO_3^- removal is not related to cytosolic Ca^{2+} levels because neither the presence nor absence of HCO_3^- or Na^+/HCO_3^- modulate cytosolic Ca^{2+} after alkalinisation by NH_4Cl (results not shown). Ca^{2+} remains in basal concentrations as it has been demonstrated in both HMC-1 sublines treated in Na^+/HCO_3^- containing medium (Lober et al., 2008b; Pernas-Sueiras et al., 2005). Carbonic anhydrases (CAs) catalyze the reaction $H_2O + CO_2 <-> HCO_3^- + H^+$ and modulate thereby intracellular HCO_3^- and pH in different cellular models (Romero et al., 2004). In both HMC-1 sublines the inhibition of this enzyme does not modulate NH_4Cl induced alkalinisation or the subsequent degranulation. These results are not unexpected since CAs are reported to be linked to Na^+ independent anion exchangers. CA II and IV bind to AE1-3 and stimulate their transporter activity. The exchanger who modulates NH_4Cl

induced alkalinisation in HMC-1 cells seems to be strongly dependent on Na^+.

We have also checked the Cl^- dependence, as many transporters exchange HCO_3^- against Cl^-, and NPPB reduced anti-IgE-stimulated Cl^- uptake and the corresponding histamine release in rodent mast cells (Redrup et al., 1997). HMC-1 cells express CIC3 and CIC5 chloride channels, which regulate a strong outwardly rectifying voltage-dependent Cl^- current (Duffy et al., 2001). The chloride channel blocker tamoxifen is able to reduce this outward Cl^- current but this does not reduce alkalinisation by NH_4Cl nor histamine release, as it is the case of DIDS and NPPB. We conclude that the mechanism, which mediates NH_4Cl response, is Cl^- independent. This is confirmed by results obtained in saline solution without Cl^-. On the one hand in both HMC-1 sublines alkalinisation reaches the same pH levels adding $(NH_4)_2SO_4$ instead of NH_4Cl and on the other $(NH_4)_2SO_4$ induced histamine release does not differ in physiologic saline solution from that in Cl^- free medium.

The fact that alkalinisation is even lower when besides HCO_3^- as well Na^+ is absent and histamine release is almost abolished in HMC-1^{560} cells might be due to a direct inhibition of the involved transporter or to a diminished Na^+ influx that depletes the exchange activity. An important Na^+ influx and thereby pH_i modulating mechanism is the Na^+/H^+ exchanger (NHE1). There are several reports that already advice to its activity in mast cells and its involvement in exocytosis (Alfonso et al., 1994; Friis and Johansen, 1996). In this work we clearly show that NH_4Cl mediated alkalinisation is linked to NHE1 activation. NHE inhibition strongly reduces NH_4Cl induced alkalinisation in HMC-$1^{560,816}$ cells. It is to bear in mind that PKC has been described to increase the affinity of NHE for Na^+ and enhance its exchange activity. Our study supports previous reports in which it was suggested that PKC stimulation might affect the action of NH_4Cl on HMC-1^{560} cells by enhancing NHE activity (Pernas-Sueiras et al., 2006a). It might be that in those studies NHE1 was activated by PKC and therefore NH_4Cl induced alkalinisation and the histamine release increased. Also alkalinisation evoked by ionomycin in HMC-1 cells might be considered to be NHE1 regulated as its effects are

as well elicited by PKC stimulation. In this context much work could be done to enclose the role of NHE1 in HMC-1 exocytosis. In this work we conclude that alkalinisation and the subsequently induced degranulation is regulated at least by a Na^+/HCO_3^- exchanger which is independent of Cl^-.

Another interesting aspect of our study is the different sensitivity that demonstrate HMC-1^{560} and HMC-$1^{560,816}$ cells concerning their activation. While histamine release is almost abolished in HMC-1^{560} cells in Na^+/HCO_3^- free conditions, the other subline only shows a diminished histamine release. This might be due to a different expression of the involved HCO_3^- exchanger in both sublines as it is reported in tumoral cells. For example the NBCn1 is higher expressed in cancer cells and as HMC-$1^{560,816}$ proliferate more rapidly they might be considered as more active (Sundstrom et al., 2003). Another possibility is that transduction pathways activated after alkalinisation which lead at the end to mast cell degranulation are different in their activation or sensitivity. We have already observed a different behaviour against other stimuli and a profound study of the role of the 816 mutation and the consequences for transduction pathways and mast cell activation might be of interest. In this context it is to bear in mind that this mutation could be identified in more than 80% of systemic mastocytosis cases and it is a minor criteria to diagnose this disease.

FIGURES

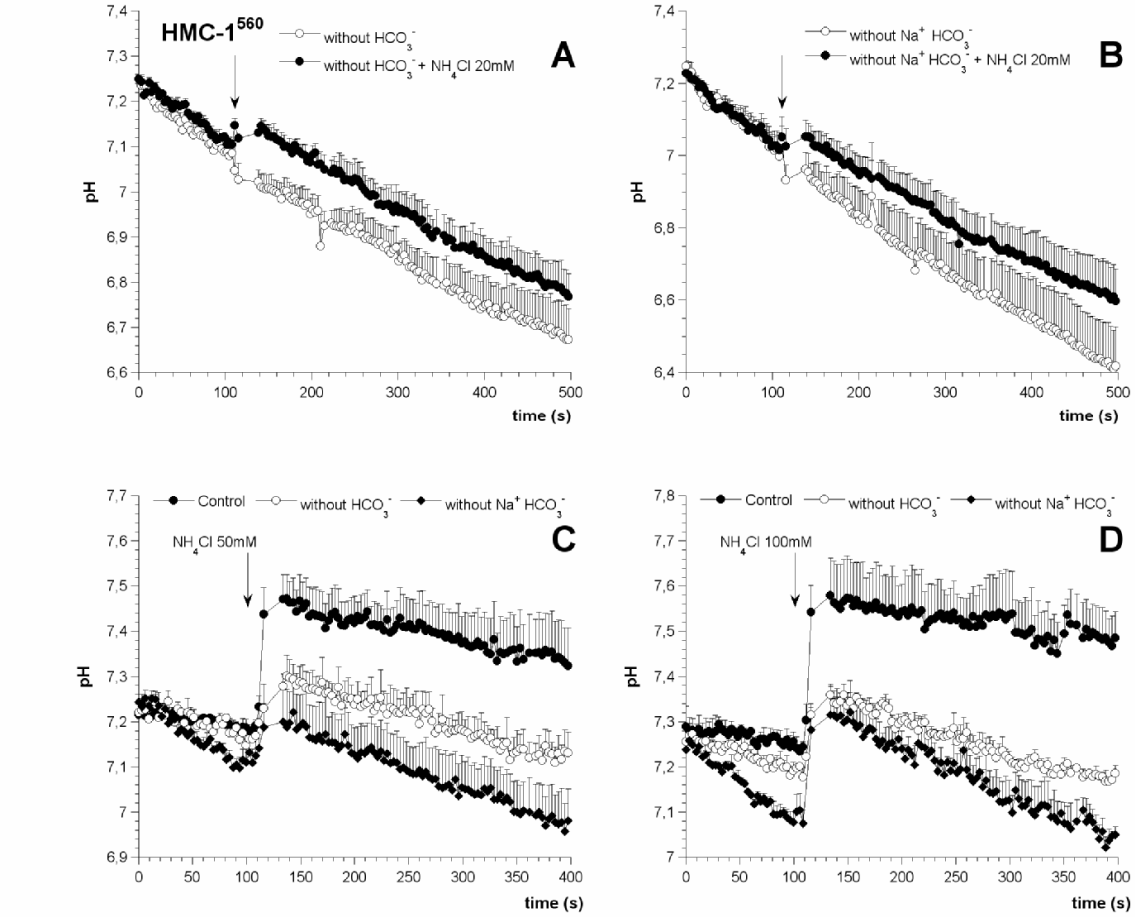

Figure 1: Effect of HCO_3^- and Na^+/HCO_3^- free saline solution on NH_4Cl-induced alkalinisation in HMC-1^{560} cells. Intracellular pH profile **(A):** NH_4Cl (20mM) was added in free saline solution. **(B):** NH_4Cl (20mM) was added in Na^+/HCO_3^- free saline solution. **(C):** NH_4Cl (50mM) was added in Umbreit, saline solution without HCO_3^- or without Na^+/HCO_3^-. **(D):** NH_4Cl (100mM) was added in Umbreit, saline solution without HCO_3^- or without Na^+/HCO_3^-. The arrows indicate the addition point. Extracellular pH=7.2 was constant during the whole experiment. Mean+/-SEM of three experiments.

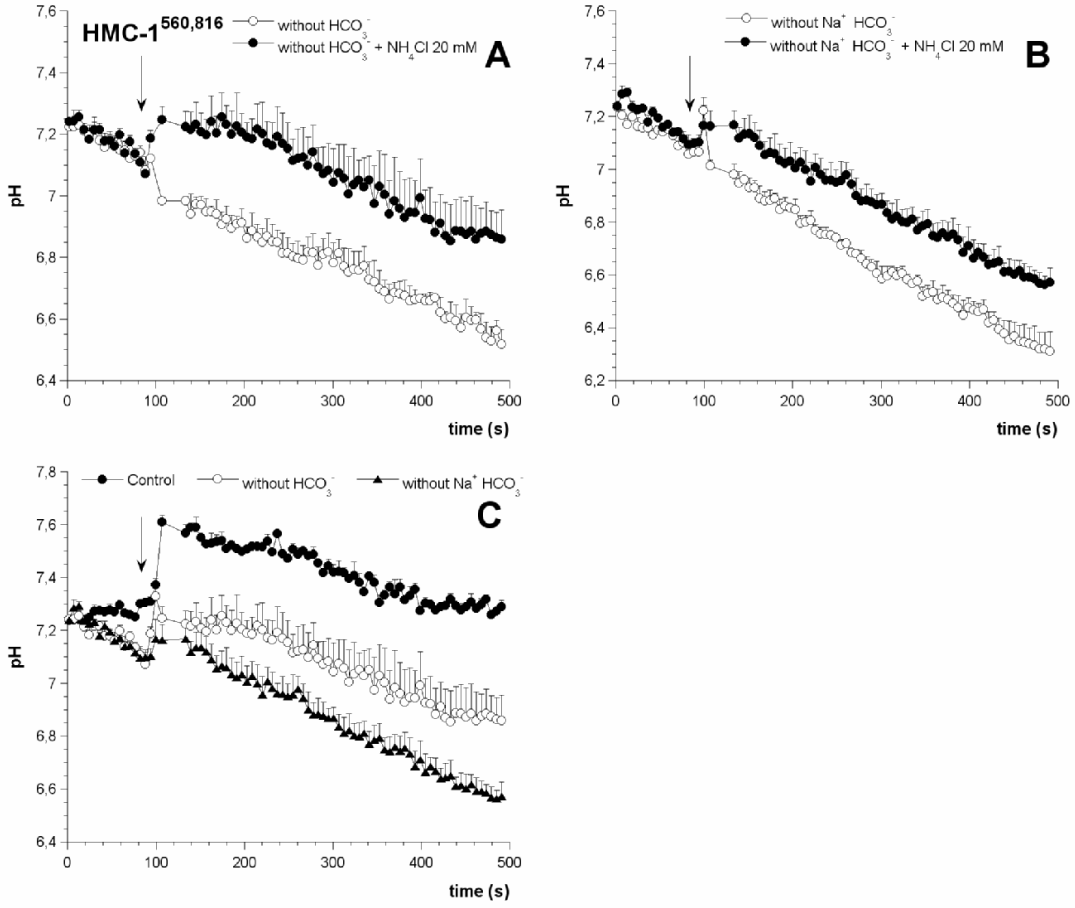

Figure 2: Effect of HCO_3^- and Na^+/HCO_3^- free saline solution on NH_4Cl - induced alkalinisation in HMC-1^{560,816} cells. Intracellular pH profile **(A):** NH_4Cl (20mM) was added in HCO_3^- free saline solution. **(B):** NH_4Cl (20mM) was added in Na^+/HCO_3^- free saline solution. **(C):** NH_4Cl (20mM) was added in Umbreit, saline solution without HCO_3^- or without Na^+/HCO_3^-. The arrows indicate the addition point. Extracellular pH=7.2 was constant during the whole experiment. Mean+/-SEM of three experiments.

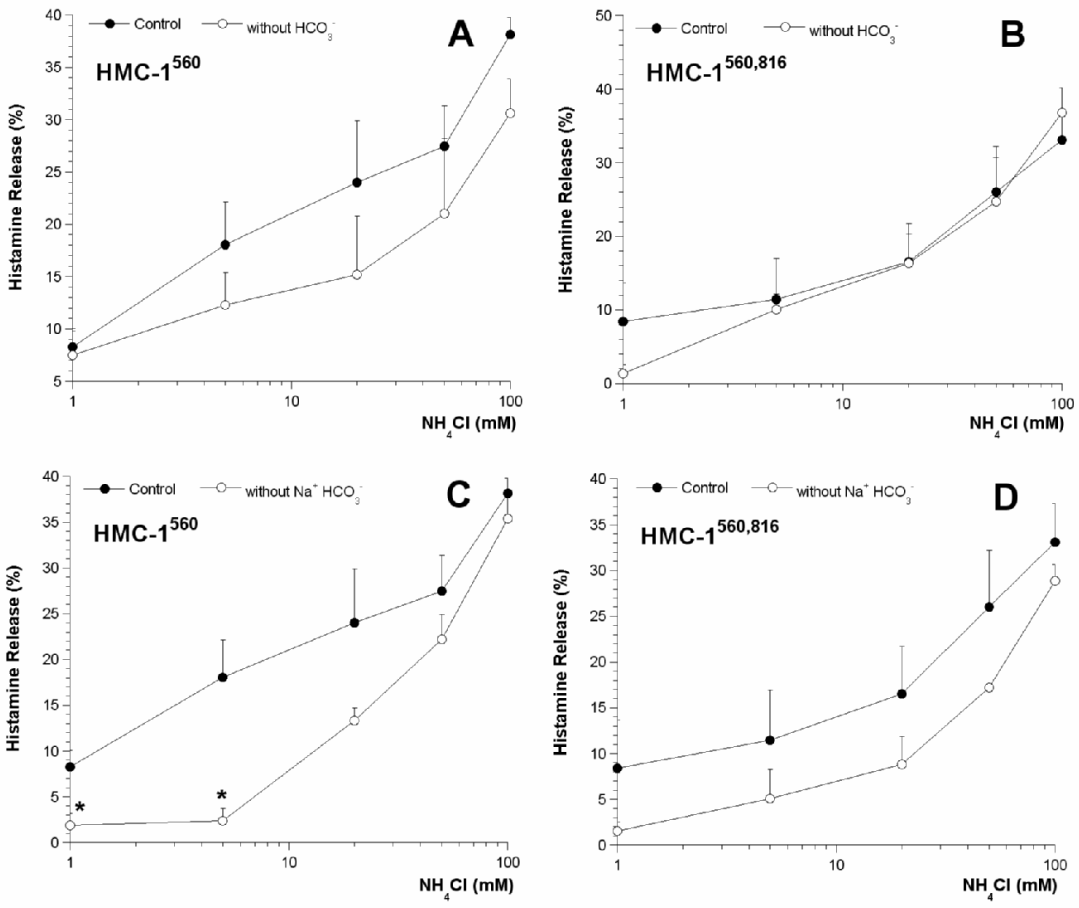

Figure 3: Effect of HCO_3^- and Na^+/HCO_3^- free saline solution on alkalinisation- induced histamine release in HMC-1^{560} and HMC-$1^{560, 816}$ cells. Histamine release profile: HMC-1^{560} cells were stimulated with different concentrations of NH_4Cl in **(A)** HCO_3^- free saline solution or in **(C)** Na^+/HCO_3^- free saline solution; HMC-$1^{560,816}$ cells were stimulated with different concentrations of NH_4Cl in **(B)** HCO_3^- free saline solution or in **(D)** Na^+/HCO_3^- free saline solution during 10 minutes at 37°C. Mean+/-SEM of three experiments. (*) Significant differences between control and drug pre-incubated cells.

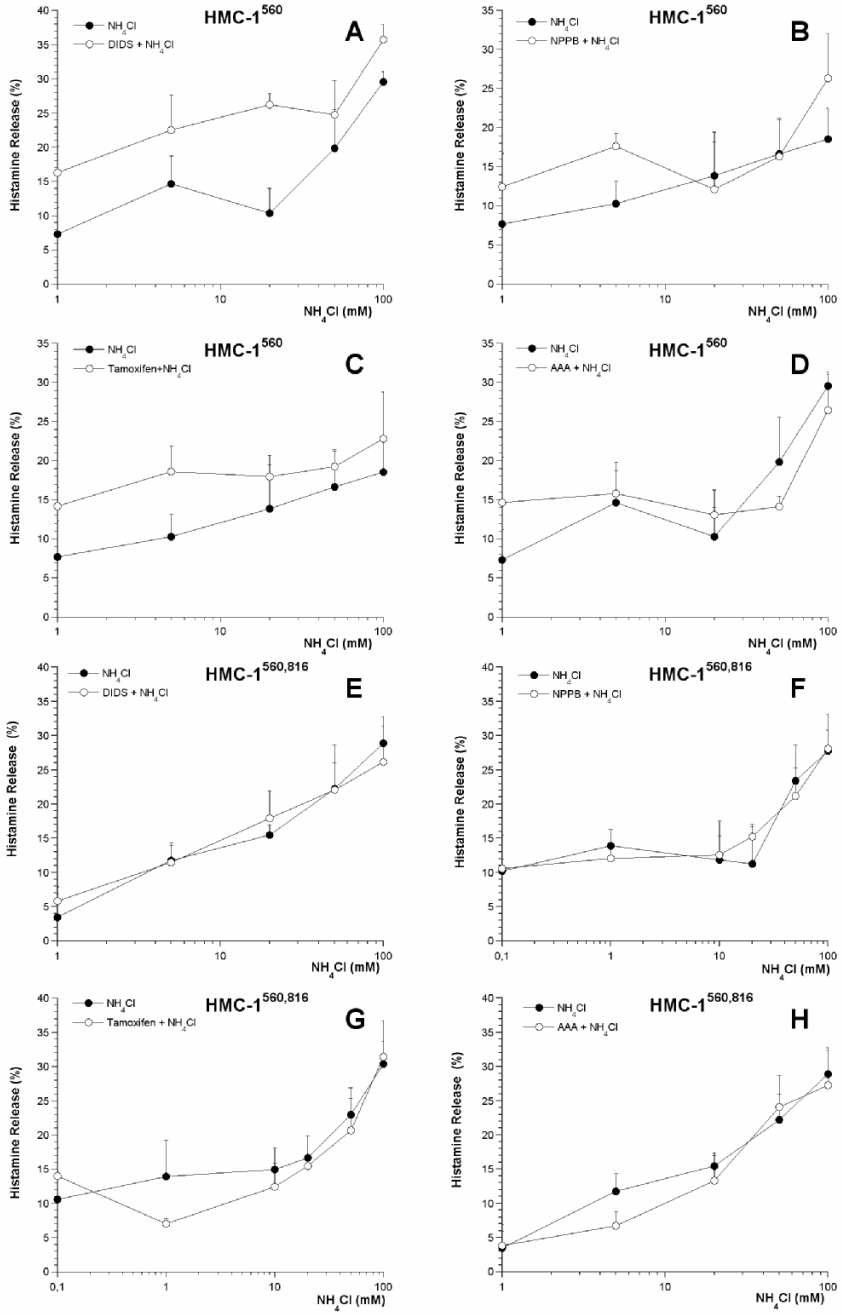

Figure 4: Effect of anion exchange modulation on alkalinisation-induced histamine release in HMC-1^{560} and HMC-1^{560,816} cells. Histamine release profile: HMC-1^{560} cells were pre-incubated during 10 minutes at 37°C with **(A)** DIDS 200µM, **(B)** NPPB 200µM, **(C)** tamoxifen 50µM or **(D)** acetazolamide (AAA) 500µM and afterwards stimulated with different concentrations of NH_4Cl in Umbreit during 10 minutes at 37°C; HMC-1^{560,816} were pre-incubated during 10 minutes at 37°C with **(E)** DIDS 200µM, **(F)** NPPB 200µM, **(G)** tamoxifen 50µM or **(H)** acetazolamide (AAA) 500µM and afterwards stimulated with different concentrations of NH_4Cl in Umbreit during 10 minutes at 37°C. Mean+/-SEM of three experiments.

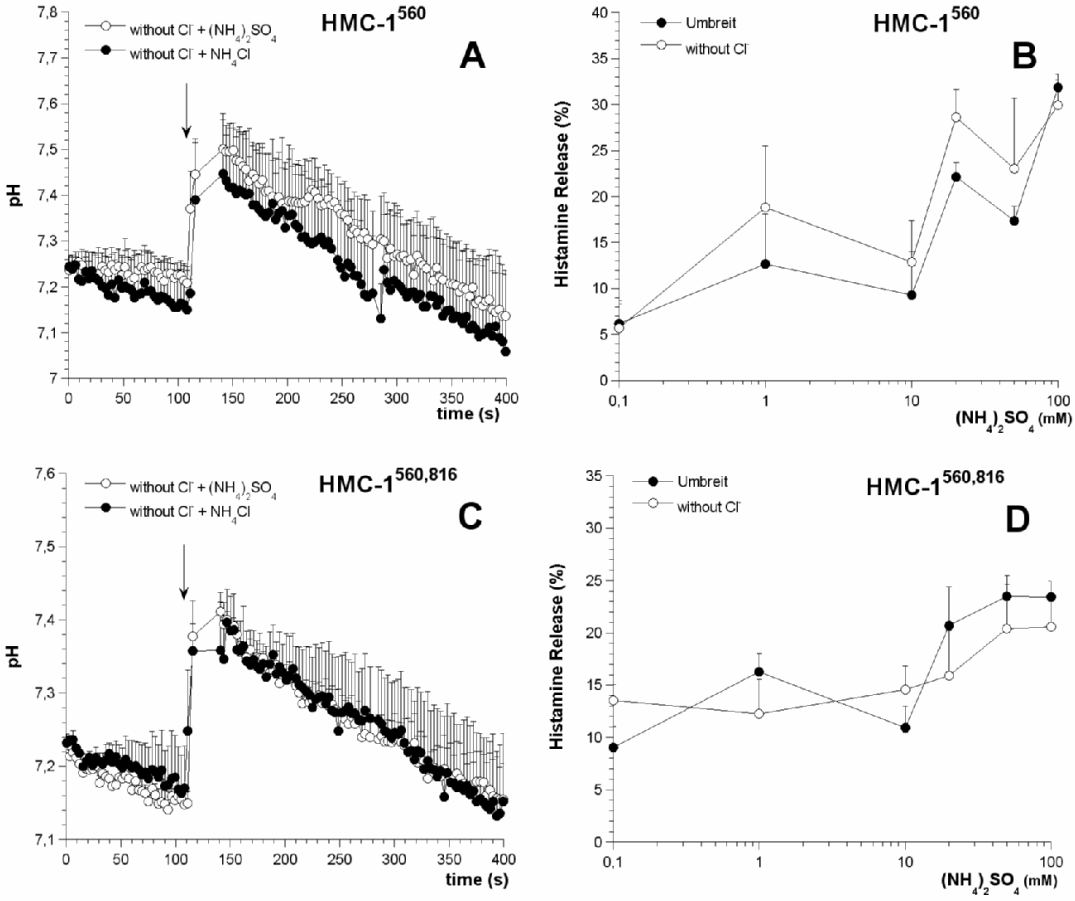

Figure 5: Effect of Cl^- suppression on NH_4^+ induced alkalinisation and histamine release in HMC-1^{560} and HMC-$1^{560,816}$ cells. Intracellular pH profile when NH_4Cl (20mM) or $(NH_4)_2SO_4$ (20mM) was added to **(A)** HMC-1^{560} cells or **(C)** HMC-$1^{560,816}$ cells in Cl^- free saline solution. Histamine release profile: **(B)** HMC-1^{560} cells or **(D)** HMC-$1^{560,816}$ cells were stimulated with different concentrations of $(NH_4)_2SO_4$ during 10 minutes at 37°C in Umbreit or in saline solution without Cl^-. Mean+/-SEM of three experiments.

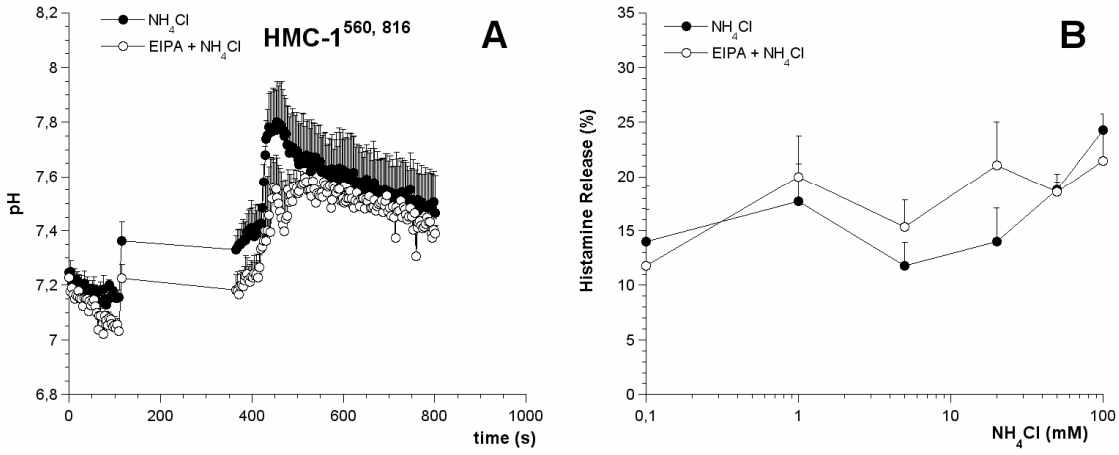

Figure 6: Effect of NHE inhibition on NH_4Cl induced alkalinisation and histamine release in HMC-$1^{560,816}$ cells. (A) Intracellular pH profile: HMC-$1^{560,816}$ cells were pre-incubated during 5 minutes with **(A)** EIPA 5µM and afterwards NH_4Cl (20mM) was added. **(B)** Histamine release profile: HMC-$1^{560,816}$ cells were pre-incubated during 10 minutes at 37°C with EIPA (5µM) and afterwards stimulated with different concentrations of NH_4Cl in Umbreit during 10 minutes at 37°C. Mean+/-SEM of three experiments.

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3.3 SECCIÓN III

III.A: PKC stimulation has different effects in human mast cells HMC-1^{560} and HMC-1^{560,816}

PKC stimulation has different effects in human mast cells HMC-1^{560} and HMC-$1^{560,816}$

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Key words: HMC-1, mast cells, protein kinase C, cytosolic calcium, Ionomycin.

ABSTRACT

The human mast cell lines HMC-1^{560} and HMC-$1^{560,816}$ were used to study their histamine response, Ca^{2+} signalling and c-kit down-regulation after Ca^{2+} ionophore or/and PKC stimulation. Both sublines carry activating mutations in the proto-oncogene of c-kit that cause autophosphorylation and permanent c-kit tyrosine kinase activation. Both have the Gly-560 -> Val mutation but only the second carries the Asp-816 -> Val mutation.
In HMC-$1^{560,816}$ cells histamine release increased after stimulation with the Ca^{2+} ionophore Ionomycin and this effect was inhibited after PKC δ stimulation. However PKC inhibition did not modify Ionomycin induced histamine release. Exocytosis evoked by Ionomycin was strictly dependent on the presence of extracellular Ca^{2+}, although in its absence cytosolic Ca^{2+} increased due to reservoir depletion. Further, a possible relationship of c-Kit down-regulation and histamine release was studied after PKC activation since PMA had an opposite effect on exocytosis in HMC-1^{560} and HMC-$1^{560,816}$ cells. Ionomycin down-regulated c-Kit expression in a dose dependent manner in HMC-1^{560} and HMC-$1^{560,816}$ cells. These results matched with the Ionomycin evoked cytosolic Ca^{2+} increase. The effect was completely abolished in Ca^{2+} free saline solution in HMC-$1^{560,816}$ cells, but not in HMC-1^{560} cells. Prior stimulation of PKC increased Ionomycin triggered response in HMC-1^{560}, but not in HMC-$1^{560,816}$ cells.
In conclusion PKC δ acts as a negative regulator of HMC-1 exocytosis induced by Ionomycin. PKC and Ionomycin down-regulation of c-kit occurs by completely different mechanisms and differs between HMC-1^{560} and HMC-$1^{560,816}$ cells. The differences might be due to the activation of distinct or further kinases. There was no relationship between histamine release and c-kit down-regulation after PMA/Ionomycin stimulation.

Abbreviations: HMC-1, Human Mast Cell Line; PKC, protein kinase C; Ca^{2+}, calcium; TyrK, tyrosine kinase.

INTRODUCTION

Mast cells are tissue-based inflammatory cells produced in the bone marrow and released after their differentiation into peripheral tissues. They are abundant in skin, thymus, and lymphoid tissue as well as around blood vessels and submucosal layer of the digestive tract. Mast cells are part of the immune system and they release vascular active substances in response to danger signals of innate or acquired immunity. One of those substances is histamine. The release of this amine can lead to reactions in the skin, like erythema and edema, in the airways, like mucous secretion and cough as well as nausea, vomiting, diarrhoea and cramping in the gastrointestinal tract. Ig-E dependent hypersensitivity or diseases of tissue disorders can evoke pathologic increases in mast cell number. The most striking increase occurs in patients with mastocytosis (Prussin and Metcalfe, 2006).

The Human Mast Cell Lines HMC-1 express in their membrane the permanent activated receptor tyrosine kinase (TyrK) c-kit (Butterfield et al., 1988). Two activating mutations in the proto-oncogene of c-kit cause its autophosphorylation and activation of the inner TyrK and induce thereby the ligand-independent proliferation of these cells (Furitsu et al., 1993; Kitayama et al., 1995; Longley et al., 2001; Ma et al., 1999). There are two HMC-1 sublines. HMC-1^{560} and HMC-$1^{560,816}$ show different behaviour concerning to drug response, phenotype and growth. Both have the Gly-560 -> Val mutation but only the second carries the Asp-816 -> Val mutation. The 560 mutation commonly appears in patients with cutaneous mastocytosis, whereas the 816 mutation can be found in 80 % of patients with systemic mastocytosis (Valent et al., 2001).

C-kit autophosphorylation in HMC-1^{560} and HMC-$1^{560, 816}$ cells leads on to a permanent activation of other transduction pathways including the Ras-MAPK-ERK and the PI3K pathways which are involved in cell survival and proliferation (Furitsu et al., 1993; Sundstrom et al., 2003). The natural ligand of c-kit is SCF (Stem Cell factor). In normal mast cells its union promotes proliferation and maturation (Tsai et al., 1991a; Tsai et al., 1991b). Further it is able to directly induce murine and human mast

cell degranulation and it potentiates Ig-E mediated histamine release in low concentrations. It is demonstrated that rhSCF induced histamine release was accompanied by an increase in cytosolic Ca^{2+} in human skin mast cells, which was inhibited by PKC activation (Columbo et al., 1994; Columbo et al., 1992; Taylor et al., 1995; Wershil et al., 1992).

Protein kinase C (PKC) is a family of Ser/Thr kinases with different isoforms, subdivided into three groups. The conventional PKCs (α, β and γ) are Ca^{2+} dependent and activated by DAG or phorbol ester; the novel PKCs (δ, ε, η and θ), which are Ca^{2+} independent, but require DAG or phorbol ester for their activation and the atypical isoenzymes (ζ, λ and ι), which seem to be independent of both factors (Lessmann et al., 2006). PKC is essential to cell function and influence different important cellular functions like inflammation, proliferation, differentiation and survival (Clemens et al., 1992; Kikkawa et al., 1986; Kikkawa and Nishizuka, 1986; Musashi et al., 2000; Nishizuka, 1984; Nishizuka, 1986; Queralt et al., 2000). For instance HMC-1 cells produce after stimulation with the phorbol ester PMA and A23187, a Ca^{2+} ionophore, amounts of interleukins and TNF-α (Zhao et al., 2004). Inhibition of production of these inflammatory substances is consistent with PKC α and δ inhibition (Sandler et al., 2005). By means of morphological analysis it was reported that shape, but not adhesion of HMC-1, is affected by PKC inhibitors (Kuchler et al., 2006). PKC stimulation can also modulate histamine release of mast cells. The best studied isoform involved in mast cell activation is PKC δ. It is highly expressed in culture and mouse mast cells and shown as a positive and also negative modulator of mast cell degranulation depending on mast cell type and stimulus (Kim et al., 1994; Leitges et al., 2002; Sandler et al., 2005). Other isoforms expressed in mast cells and involved in their activation are PKC α, β, ε y θ (Kim et al., 1994; Lessmann et al., 2006; Liu et al., 2001; Ludowyke et al., 2006; Ludowyke et al., 1996). In addition, PKC stimulation and Ca^{2+} mobilisation by ionophores are shown to down-regulate c-kit surface expression of mast cells (Babina et al., 2006; Yee et al., 1993).

In HMC-1^{560} cells PKC stimulation augmented Ionomycin induced histamine release due to an increase in cytosolic Ca^{2+} influx (Pernas-Sueiras et al., 2006). In this work we observe an opposite histamine response in HMC-$1^{560,816}$ cells, by the same treatments and we try to disclose if this difference might be based on distinct effects on PKC δ and c-kit activation.

METHODS

Chemicals

Ammonium chloride (NH_4Cl) was from Panreac (Barcelona, Spain). Rottlerin, ionomycin and GF109203X were from Alexis Corporation (Läufelingen, Switzerland). FURA-2 AM and 2,7-bis (carboxyethyl)-5(6)carboxy-fluorescein-acetoxymethylester (BCECF AM) were from Molecular Probes (Leiden, The Netherlands). Phorbol 12-myristate 13-acetate (PMA) was from Sigma-Aldrich (Madrid, Spain). Anti CD117-PE, Anti PKC δ was purchased from BD Biosciences (Madrid, Spain) and Alexafluor 546 from Molecular Probes, Carlsbad, CA, USA.

Cell cultures

HMC-1 cells were kindly provided by Dr. J. Butterfield (Mayo Clinic, Rochester, MN) and maintained in Iscove´s modified Dulbecco´s medium (IMDM) supplemented with 10 % fetal bovine serum (FBS), 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were expanded weekly.

Cell preparation

For histamine release assays, cells were centrifuged (1500 r.p.m., 5 minutes, 4 ºC) and washed twice with saline solution (1000 r.p.m., 5 minutes, 4 ºC). The composition of this solution was (mM): Na^+ 142.3; K^+ 5.94; Ca^{2+} 1; Mg^{2+} 1.2; Cl^- 126.2; HCO_3^- 22.85; HPO_4^{2-} 1.2, SO_4^{2-} 1.2; glucose 1 g/l.

For Ca^{2+} and pH measurements cells were treated in the same conditions, but washed in saline solution plus 0.1 % bovine serum albumin (BSA).

The incubation medium was equilibrated with CO_2 prior to use. During the experiments, pH (7.20) was maintained constant by bubbling CO_2. Experiments were carried out at least three times, by duplicate, both for histamine release assays and Ca^{2+} and pH measurements.

Histamine release assays

6.2 µl of a freshly prepared concentrated solution of each drug were added to the incubation medium to attain a final volume of 150 µl and pre-incubated. When the medium reached 37 ºC, 100 µl of a cell suspension with an approximate density of 1.5-2 x 10^6 cells/ml were added to each tube. Incubations were carried out in a bath at 37 ºC for 10 minutes. The incubations were stopped by immersing the tubes in a cold bath. After centrifugation at 2300 r.p.m. for 10 minutes, the supernatants were collected and decanted into other tubes for histamine determination. Appropriate controls to determine spontaneous histamine release in the absence of stimuli were executed in every experiment.

Histamine release was tested with a spectrofluorometer, (Fluoroskan II, Labsystems, Barcelona, Spain) in both pellet (non-released histamine) and supernatant (released histamine) according to Shore´s method (Shore, 1971 13). To produce the fluorescent complex 0.04% orthophthaldialdehyde was used, also trichloroacetic acid (14 %) to avoid protein interferences in the histamine release determination. To ensure total histamine, pellets were sonicated for 60 seconds in 0.2 ml of 0.1 N HCl. Results shown were expressed as the percentage of released histamine from the total histamine content.

Cytometry

Cells were treated like in histamine assays. After washing they were incubated for 30 minutes with human anti CD117-PE (BD Biosciences) in saline solution with BSA 1% and analysed in a flow cytometer.

Measurement of cytosolic free Ca^{2+} and intracellular pH

HMC-1 cells were loaded with FURA-2 AM (0.2 μM) and BCECF AM (0.05 μM) in a bath at 37 ºC, for 10 minutes. After this time, loaded cells were washed with saline solution (1000 r.p.m., 10 minutes, 4 ºC). Cells were attached to glass coverslips treated with poly-L-lysine, and these were inserted into a thermostated chamber (Life Sciences Resources, U.K.). Cells were viewed using a Nikon Diaphot 200 microscope equipped with epifluorescence optics (Nikon 40x– immersion UV– Fluor objective). Addition of drugs was made by aspiration and addition of fresh bathing solution to the chamber. Cytosolic Ca^{2+} concentrations were obtained from the images collected by fluorescence equipment (Life Sciences Resources, U.K.). The light source was a 175 W xenon lamp, and the used wavelengths were selected with filters. For FURA-2 AM, the excitation wavelengths were 340 and 380 nm, with emission at 505 nm. The calibration of the fluorescence values *vs.* intracellular Ca^{2+} was made according to the method of Grynkiewicz (Grynkiewicz et al., 1985). For BCECF AM the excitation was performed at 440 and 490 nm, with 530 nm for emission. The calibration fluorescence values *vs.* pH as per Thomas *et al*. (Thomas et al., 1979). In brief, a calibration curve was obtained with four known values of pH, measuring the fluorescence ratio obtained in the presence of nigericin, in a K^+ solution, for each pH value.

Immunocytochemistry and Confocal Microscopy

Control and treated cells were attached to glass coverslips treated with poly-L-lysine, washed with PBS and fixed with formaldehyde (4 %). Subsequently they were labelled with a primary antibody against PKC δ (1:1000, BD Biosciences) for 24 hours. The preparation was washed three times with PBS and then exposed to the secondary antibody Alexafluor 546 (1:1000) for 2 hours. The fluorescent image was viewed with a laser-scanning confocal microscope (Nikon, Mellville, NY, USA), with a Hamamatsu ORCA-ER camera (Hamamatsu Photonics KK, Hamamatsu, Japan).

Western blotting

Cells were incubated during 10 minutes in Umbreit solution or in Na^+/HCO_3^- free solution. Afterwards cells were lysed, centrifuged and the supernatant was blotted by reduced SDS-PAGE.

After one hour of blockage with 5% non-fat dry milk the membrane was incubated overnight with anti-PKC δ (1:500, BD Biosciences). After four washes with washing buffer the membrane was incubated for two hours with secondary peroxidase-labelled antibody (Amersham). After four washes chemiluminescence was visualized with SuperSignal® West Pico (Pierce). Relative protein expression was calculated in relation to β-tubulin (Sigma-Aldrich) expression for each experiment. Experiments were carried out three times.

Statistical analysis.

Results were analyzed using the Student's t-test for unpaired data. A probability level of 0.05 or smaller was used for statistical significance. Results were expressed as the mean ± SEM.

RESULTS

It has been described that exocytosis of rat mast cells and human mast cells (HMC-1) can be stimulated by the Ca^{2+} ionophore Ionomycin. This histamine release was even higher when PKC was activated by phorbol esters (Alfonso et al., 2000; Pernas-Sueiras et al., 2005). Phorbol 12-myristate 13-acetate (PMA) activates PKC by linking to the DAG binding site. Unlike DAG, PMA is not metabolized and has a longer effect. Its binding activates and translocates the enzyme to the membrane where it phosphorylates subsequent enzymes like MAP-kinases, Raf-kinase, transcription factor inhibitor or epidermal growth factor receptor. Thus we pre-incubated HMC-$1^{560,816}$ cells with the phorbol ester PMA (100 ng/ml) and then the cells were stimulated with different concentrations of Ionomycin. As Fig. 1A shows, the pre-incubation of HMC-$1^{560,816}$ cells with PMA significantly decreased Ionomycin-induced histamine release in concentrations up to 10 μM of the ionophore. In order to prove the Ca^{2+}

dependence of histamine release evoked by the ionophore, cells were incubated in presence and absence of extracellular Ca^{2+}. As Fig. 1B shows, exocytosis was completely abolished when Ca^{2+} was absent. Therefore we proved if the inhibiting effect of PMA could be due to changes in cytosolic Ca^{2+} levels. Experiments performed in similar conditions as histamine assays demonstrated that cytosolic Ca^{2+} increased at the addition point of Ionomycin (0,1 μM), Fig. 2A, due to the depletion of intracellular Ca^{2+} reservoirs (125 nM) and decreased steadily afterwards. Ca^{2+} profile raised again to values about 500 nM when Ca^{2+} was restored to the extracellular medium. The same figure shows that PMA (100 ng/ml) addition alone did not affect cytosolic Ca^{2+} levels; in contrast when cells were pre-treated with PMA prior to Ionomycin (0,1 μM), Ca^{2+} reservoir depletion was lower by 20 nM with respect to Ionomycin alone, and Ca^{2+} influx was significantly higher (150 nM higher than Ionomycin alone). The decreasing effect of PMA on intracellular reservoir depletion was more evident using 1 μM of Ionomycin (47 nM lower than Ionomycin alone), Fig. 2B, whereas the enhancing effect on Ca^{2+} influx disappeared. Ionomycin (1 μM) alone induced higher reservoir depletion but not higher Ca^{2+} influx (500 nM). The inhibition of reservoir depletion by PKC activation was even more notable using 10 μM of Ionomycin (86 nM lower than Ionomycin alone; Fig 2C). Ca^{2+} influx reached values around 520 nM and no differences could be observed between PMA treated and control cells. Results of these experiments are shown in Fig. 2D and it can be observed that the phorbol ester significantly inhibited intracellular Ca^{2+} reservoir depletion induced by different concentrations of Ionomycin. There were no significant intracellular pH changes in the same experiments (results not shown). Therefore, from these results, cytosolic Ca^{2+} increase after reservoir depletion was not sufficient signal to induce exocytosis (Fig. 1B).

In order to check the effect of PKC inhibition on histamine response of HMC-$1^{560,816}$ cells two inhibitors were tested. Rottlerin is shown to inhibit PKC by competing with ATP but its specificity for PKC δ is controversial and being discussed (Alonso et al., 2008; Davies et al., 2000;

Gschwendt et al., 1994; Leitges et al., 2001; Tapia et al., 2006). Another PKC inhibitor is the bisindolylmaleimide GF109203X. Its inhibitory effect is also based on ATP competition with its protein binding site; its efficacy is shown on different PKC isoenzymes and depends on the concentration used (Martiny-Baron et al., 1993; Toullec et al., 1991). Fig. 3A demonstrates that pre-incubation of HMC-$1^{560,816}$ cells with Rottlerin (10 µM) did not alter histamine release induced by Ionomycin. As well cytosolic Ca^{2+} profile was observed after PKC inhibition since Rottlerin completely inhibited Ca^{2+} influx induced by Ionomycin in HMC-1^{560} cells (Pernas-Sueiras et al., 2005). Rottlerin significantly inhibited intracellular Ca^{2+} reservoir depletion and significantly reduced Ca^{2+} influx from the extracellular medium in HMC-$1^{560,816}$ cells (Fig. 3B). Further experiments were performed with GF109203X (Toullec et al., 1991). Pre-incubation with GF109203X (500 nM) did not significantly change Ionomycin induced histamine release (Fig. 3C) and did not modify cytosolic Ca^{2+} levels in HMC-$1^{560,816}$ cells (Fig. 3D). No significant intracellular pH changes were observed in the same experiments (results not shown).

Since we suggested differences in PKC δ activation between HMC-1^{560} and HMC-$1^{560,816}$ cells we performed immunocytochemistry assays after PMA pre-incubation. Control and PMA (100 ng/ml) treated cells were incubated for 10 minutes, as for histamine release experiments, and afterwards fixed and stained with PKC δ antibody. The results in Fig. 4 demonstrate that PMA activated PKC δ in both HMC-1 sublines. As Fig. 4A shows, PKC δ is distributed in the cytosol in HMC-1^{560} control cells. After stimulation by PMA, Fig. 4B, PKC δ translocated and fluorescent intensity decreased. Fig. 4C shows the distribution of PKC δ with its fluorescent intensity obtained in non-treated HMC-$1^{560,816}$ cells. Fig. 4D clearly demonstrates how fluorescent intensity decreased after PMA treatment. In HMC-1^{560} cells (Fig. 4E) fluorescence decreased about 44% from 887 to 496 units. Similar results were obtained with HMC-$1^{560,816}$ cells (Fig. 4F) with an fluorescent decrease of about 52% from 1296 to 620 units. As there were no differences between both sublines after PKC activation, cytosolic PKC δ concentration was checked in cells treated

with PMA in combination with Ionomycin by Western blotting. Fig. 5A shows the relative expression of PKC δ in the cytosolic fraction of lysed HMC-1^{560} cells. The results demonstrate that PMA treatment decreased cytosolic PKC δ expression probably due to its translocation from the cytosol to the membrane, which signifies its enzymatic activation (Ohmori et al., 1998). The same Fig. demonstrates that Ionomycin and the combination of PMA with Ionomycin did not modify cytosolic PKC δ expression. The same experiments were provided with HMC-$1^{560,816}$ cells (Fig. 5B). In these cells PMA, Ionomycin and the combination PMA with Ionomycin decreased cytosolic PKC δ expression, whereby the effect was significant in PMA and PMA/Ionomycin lysates. The differences between PMA and PMA/Ionomycin treated cells were not significant and do not let us conclude a further effect of Ca^{2+} on PKC δ activity. Fig. 5C (HMC-1^{560} cells) and Fig. 5D (HMC-$1^{560,816}$ cells) show Western blots of one representative experiment of three.

In some cases mast cell exocytosis takes place or is forced by c-kit stimulation and c-kit expression can be down-regulated by PMA stimulation (Babina et al., 2006; Columbo et al., 1994; Columbo et al., 1992; Taylor et al., 1995; Wershil et al., 1992; Yee et al., 1993). Therefore flow cytometry studies were provided in order to find out if PKC/Ca^{2+} mobilisation affects c-kit expression in HMC-1 cells and this might modulate histamine release. Fig. 6A shows that Ionomycin decreased c-kit expression in the membrane of HMC-1^{560} cells, whereas only the highest concentration had a significant effect. In contrast c-kit down-regulation in HMC-$1^{560,816}$ cells was dose-dependent and significant in every Ionomycin concentration (Fig. 6B). Fig. 6C shows that PKC stimulation of HMC-1^{560} cells by PMA alone induced significant down-regulation of c-kit whereas it does not produce any changes in cytosolic Ca^{2+} levels (Pernas-Sueiras et al., 2006). Furthermore observing PMA/Ionomycin treated cells, c-kit was significantly more down-regulated than in cells treated with Ionomycin alone. As well in HMC-$1^{560,816}$ cells, Fig. 6D, PKC activation by PMA alone significantly reduced c-kit expression, but there were no significant differences between cells stimulated by PMA/Ionomycin in combination or by the ionophore alone.

In addition as Fig. 6C (HMC-1^{560} cells) and Fig 6D (HMC-$1^{560,816}$ cells) demonstrate, in both sublines Ionomycin in high concentrations increased the effect of PMA on c-kit down-regulation. Our results indicate that the effects of PMA and Ionomycin on c-kit expression are not in correlation with histamine release and that both compounds act by distinct mechanisms on c-kit. This could be confirmed by experiments in extracellular medium without Ca^{2+}. Fig. 7A shows that PMA and the combination PMA/Ionomycin were able to significantly down-regulate c-kit expression in HMC-1^{560} cells in the absence of extracellular Ca^{2+}. In the same way Ionomycin reduced c-kit expression, but the effect was not significant with respect to its control in Ca^{2+} free medium. In HMC-$1^{560,816}$ cells, Fig. 7B, the ionophore did not show any effect when extracellular Ca^{2+} was absent. It can be concluded that Ca^{2+} increase by reservoir depletion is not a sufficient signal to down-regulate c-kit in HMC-$1^{560,816}$ cells. The same Fig. shows that PKC stimulation with and without Ionomycin treatments significantly reduced c-kit expression, probably mainly due to the effect of PMA alone. A surprising result was obtained comparing the effects of PMA with those of PMA/Ionomycin in Ca^{2+} free conditions. The effects in both sublines, Figs. 7A y B, were completely reversed to results obtained in Ca^{2+} containing medium. The effect of PMA on Ionomycin induced c-kit down-regulation was not significant in HMC-1^{560} cells but it became significant in HMC-$1^{560,816}$ cells due to the complete ineffectiveness of Ionomycin in these cells without extracellular Ca^{2+}. However a significant difference can be observed between HMC-1^{560} cells treated with PMA alone in the absence or presence of extracellular Ca^{2+} (Fig. 7C). This effect might be due to the activation of another PKC, which is dependent on the influx of Ca^{2+} from extracellular. No significant differences were detected between cells treated with PKC/Ionomycin or Ionomycin alone with respect to the presence or absence of extracellular Ca^{2+}. In contrast in HMC-$1^{560,816}$ cells, as Fig. 7D shows, down-regulation of c-kit by Ionomycin was significantly different between cells in Ca^{2+} free medium or in its presence. Whereas c-kit expression decreased about 30% in Ca^{2+} containing medium, there was absolutely no effect in the absence of the

cation. These results clearly demonstrate that this process in HMC-$1^{560,816}$ cells strictly depends on the influx of Ca^{2+} from extracellular.

DISCUSSION

The Human Mast Cell Line (HMC-$1^{560,816}$) was used to study the effect of PKC modulation and to compare this later on with previous and recent studies in HMC-1^{560} cells.

PKC influences a variety of important cell processes like morphology, proliferation, apoptosis and production of inflammatory substances in mast cells (Kuchler et al., 2006; Sandler et al., 2005; Zhao et al., 2004). Histamine release of HMC-1^{560} cells was induced by Ionomycin; prior PKC stimulation caused an increase of transmitter release due to elevated cytosolic Ca^{2+} influx (Pernas-Sueiras et al., 2006). In contrast in this study PKC activation negatively affected Ionomycin induced histamine release in HMC-$1^{560,816}$ cells and intracellular Ca^{2+} reservoir depletion. These results were surprising and inspired further investigation about the effect of PKC stimulation in HMC-1 sublines.

PKC δ is one of the mostly reported PKC isoform in connection with mast cell activation and degranulation. In RBL-2H3 mast cells antigen induced degranulation takes place by a cytosolic Ca^{2+} increase through a PKC δ-dependent pathway. Similar results were obtained when transmitter release was induced by Ca^{2+} mobilisation with an ionophore (Cho et al., 2004). Although PKC δ stimulation was suggested to facilitate mast cell degranulation, it is reported that PKC δ could act as well as a negative regulator of antigen-induced mast cell exocytosis in bone marrow-derived mast cells after studies with PKC δ negative cells (Leitges et al., 2002). Also in eosinophils PKC δ activation inhibited degranulation through stimulation of intracellular cAMP production (Ezeamuzie and Taslim, 2004). In summary PKC activation can activate and inactivate exocytosis depending on mast cell type and stimulus. In both HMC-1 sublines we could demonstrate that PMA treatments translocate PKC δ from the cytosol, probably to the membrane, which induces its enzymatic activity. In contrast the co-treatment PMA/Ionomycin only

leads to PKC δ translocation in HMC-$1^{560,816}$ cells, but not in HMC-1^{560} cells. The inactivation of PKC δ despite PMA treatment in HMC-1^{560} cells might be due to the activation of other kinases or lipases by Ionomycin induced Ca^{2+} increase. Respect to histamine release our results clearly demonstrate that PKC δ acts as a negative regulator of exocytosis in HMC-1 cells. In HMC-1^{560} cells PKC δ was inactivated after PMA/Ionomycin treatments and histamine release significantly increased. In good agreement exocytosis was significantly diminished when PKC δ was activated after PMA/Ionomycin treatments in HMC-$1^{560,816}$ cells. The negative effect of PKC δ on histamine release might be in parts regulated by modulation of Ca^{2+} reservoir depletion since this was significantly decreased with all concentrations of Ionomycin after PKC stimulation in HMC-$1^{560,816}$ cells. Rottlerin also reduces Ca^{2+} reservoir depletion and influx but does not modify histamine release, probably because it acts by a different transduction pathway and activates thereby other mechanisms (Alonso et al., 2008). Compounds used in this study modify different biochemical parameters and we have only observed two of them, cytosolic Ca^{2+} and intracellular pH. It is to note that the Ca^{2+} ionophore A23187 opens a Ca^{2+} activated Cl^- channel in HMC-1 cells; this lead to a membrane potential fall, which might affect histamine release (Duffy et al., 2001; Duffy et al., 2003).

Mast cells express in their membrane the tyrosine kinase receptor c-kit, which is involved in important processes like proliferation, activation and maturation. HMC-1^{560} and HMC-$1^{560,816}$ cells differ in activating mutations in the proto-oncogene c-kit, which cause in both sublines autophosphorylation and permanent activation of the inner TyrK and activate thereby subsequent pathways (Furitsu et al., 1993; Kitayama et al., 1995; Longley et al., 2001; Ma et al., 1999; Sundstrom et al., 2003). It has been described that activation of c-kit induces or forces mast cell exocytosis and PKC stimulation by PMA down-regulates c-kit expression in the mast cell membrane (Babina et al., 2006; Columbo et al., 1994; Columbo et al., 1992; Taylor et al., 1995; Wershil et al., 1992; Yee et al., 1993). Therefore flow cytometry studies were provided in order to find out if PKC/Ca^{2+} mobilisation affects c-kit expression in

HMC-1 cells and this might correlate with histamine release. In this study we clearly demonstrate that short-time stimulation with PMA and/or Ionomycin dose-dependently down-regulates c-kit expression in both HMC-1 sublines. Whereas distinct concentrations of Ionomycin produce significant differences in reservoir depletion and Ca^{2+} influx in HMC-$1^{560,816}$ cells and decrease thereby significant c-kit expression, these differences were not that pronounced in HMC-1^{560} cells (Lober et al., 2008b; Pernas-Sueiras et al., 2005). However Ca^{2+} increase evoked by reservoir depletion alone does not significantly affect c-kit expression, although in HMC-1^{560} cells it down-regulates slightly. It is necessary to bear in mind that also histamine release is abolished in medium without Ca^{2+}. In contrast PMA alone induces in both sublines significant down-regulation of c-kit in the presence or absence of extracellular Ca^{2+} without producing any changes in cytosolic Ca^{2+} levels, but it does not affect histamine release (Pernas-Sueiras et al., 2006). Here it is clearly demonstrated that c-kit down-regulation by PMA and Ionomycin takes place by completely different and independent mechanisms. Whereas the effect of Ionomycin on c-kit is completely dependent on the influx of Ca^{2+}, the effect of PMA seems to be Ca^{2+} independent. Therefore we suppose that the PKC involved in this process might be one of the novel isoformes like PKC δ, which is activated in both sublines after PMA treatment. Differences between HMC-1^{560} and HMC-$1^{560,816}$ cells were obtained observing the effect of PKC activation on Ionomycin induced down-regulation. Whereas HMC-1^{560} cells additionally decrease c-kit expression after Ionomycin treatment, there were no differences in HMC-$1^{560,816}$ cells. In exocytosis, these results indicate that c-kit expression does not directly correlate with histamine release. In HMC-$1^{560,816}$ cells results from PMA/Ionomycin treatments do not differ to those from Ionomycin alone concerning c-kit down-regulation, but histamine release is significantly diminished in PMA pre-treated cells. It seemed that there is another Ca^{2+} dependent PKC involved in the process of c-kit regulation in HMC-1^{560} cells. This becomes clearer in experiments in the absence of extracellular Ca^{2+} showed in the last figure. Our results demonstrate on the one hand a significant difference between PMA treated HMC-1^{560} cells

with and without Ca^{2+}, which might be due to the inhibition of a Ca^{2+} dependent PKC in Ca^{2+} free conditions. On the other hand there is a significant difference between Ionomycin treated HMC-$1^{560,816}$ cells with and without Ca^{2+} due to the complete ineffectiveness of the compound in the absence of the ion. We do not observe this difference in HMC-1^{560} cells, maybe due to the activation of a Ca^{2+} dependent PKC, activated by the Ca^{2+} increase after reservoir depletion. Further the effects of PMA/Ionomycin were completely reversed compared to results obtained in Ca^{2+} containing medium. Whereas in HMC-1^{560} cells down-regulation of c-kit expression was significant different between Ionomycin and PMA/Ionomycin treated cells, this effect disappeared in the absence of Ca^{2+}. In contrast HMC-$1^{560,816}$ down-regulation of c-kit was not significantly different between Ionomycin and PMA/Ionomycin treated cells. In the absence of Ca^{2+} it became significant due to ineffectiveness of Ionomycin in these conditions. The results let suggest that there is another PKC involved in PMA and Ionomycin induced c-kit down-regulation in HMC-1^{560} cells, which is not activated in HMC-$1^{560,816}$ cells.
Differences between HMC-1^{560} and HMC-$1^{560,816}$ cells have been already described with regards to drug response, phenotype, growth and even PKC effects concerning to mast cell adhesion (Lober et al., 2008a; Lober et al., 2008b; Sundstrom et al., 2003). In one study, cells carrying the wild-type murine c-kit were compared with an Asp-814->Tyr mutant in a murine mast cell-like line. PKC δ was constitutive activated, lipid-dependent and Ca^{2+} independent. The kinase demonstrated higher activity in the mutant type than in the wild type. A mutation in human c-kit codon 816 was identified in HMC-$1^{560,816}$ cells and corresponds to the murine codon (Asp-814) (Jelacic and Linnekin, 2005; Linnekin, 1999). In this work, also differences in histamine release and down-regulation of c-kit expression were observed after PKC stimulation. It might be that these differences between HMC-1^{560} and HMC-$1^{560,816}$ cells are a hint of the distinct PKC effect like it has been found in Asp-814 mouse mutants. In the corresponding mouse model higher levels of PKC δ activity were not mediated by higher amounts of PKC δ protein. It might be that the mutation in position 816 that carry HMC-$1^{560,816}$ cells, but not HMC-1^{560}

cells, modifies the substrate specificity of PKCs and thereby changes the effects of subsequent enzymes and results in different cell responses after same stimulation.

In summary, results shown in this work indicate that PKC δ is activated by phorbol esters in HMC-1^{560} and HMC-$1^{560,816}$ cells. They demonstrate that PKC δ is a negative regulator of exocytosis in these cells. Down-regulation of c-kit by PKC activation is not correlated to the process which leads to degranulation. Further this study is another hint that HMC-1^{560} and HMC-$1^{560,816}$ are two distinct cultured human mast cell lines (Sundstrom et al., 2003). Down-regulation of one of the most important mast cell receptors, c-kit, seems to be regulated by different kinases in both HMC-1 sublines and this should be related to their mutations.

FIGURES

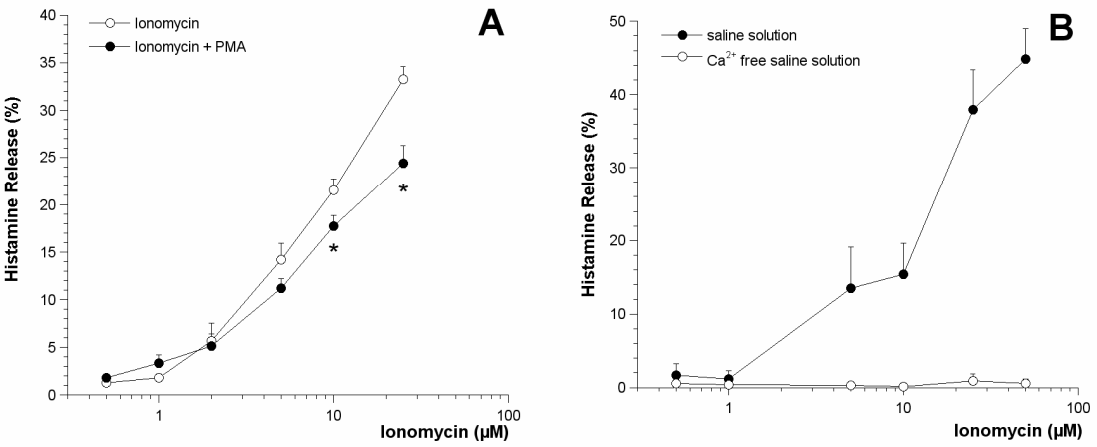

Figure 1: Effect of PKC stimulation and extracellular Ca^{2+} on histamine release of HMC-1^{560,816} cells. Histamine release profile of HMC-1^{560,816} cells: **(A)**: Pre-incubation during 10 minutes with PMA 100 ng/ml and afterwards stimulated with different concentrations of Ionomycin for 10 minutes at 37°C. **(B)**: Incubation with different concentrations of Ionomycin for 10 minutes in saline solution with and without Ca^{2+} at 37°C. Mean+/-SEM of three experiments. (*) Significant differences between Ionomycin and PMA pre-treated cells.

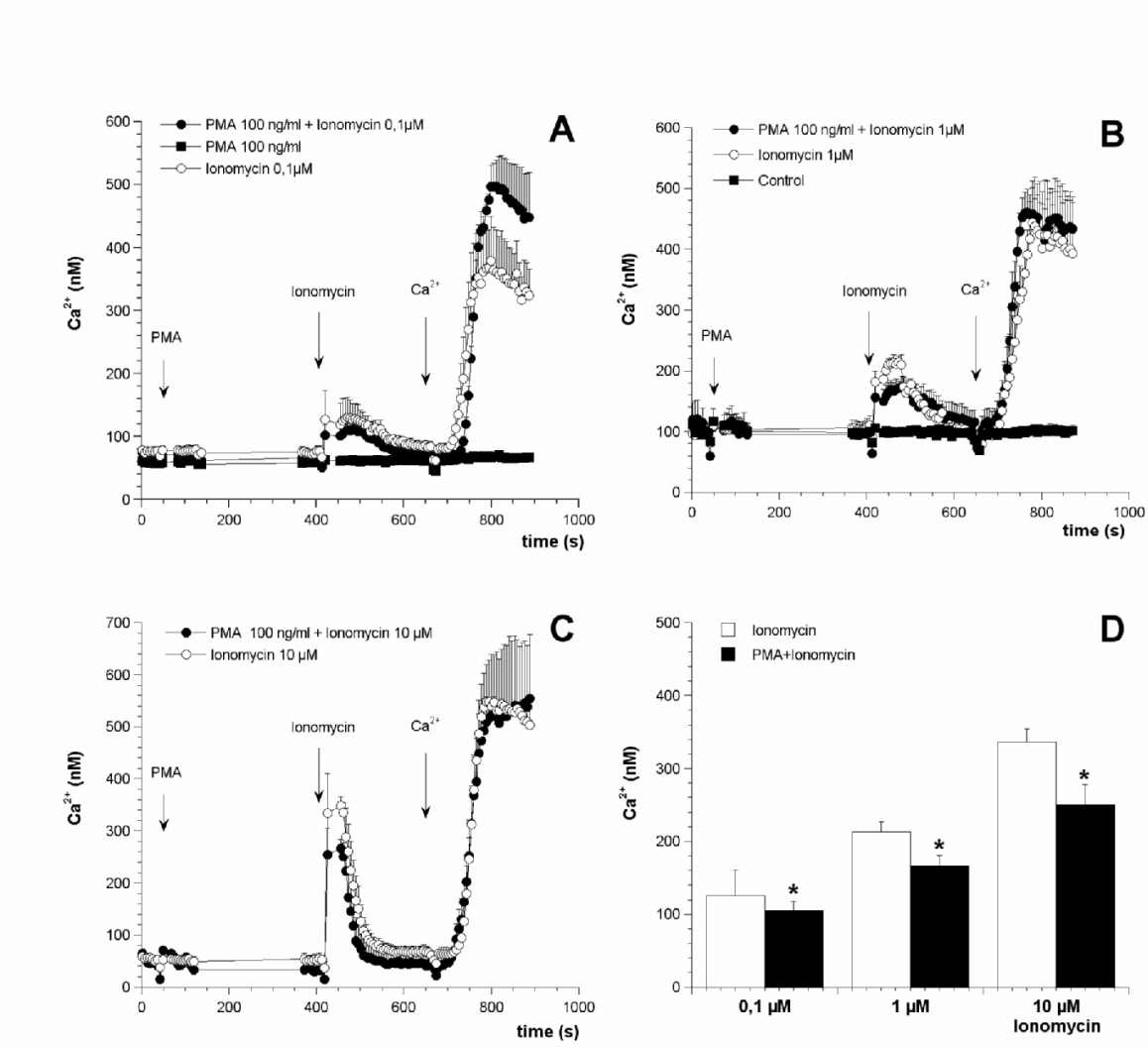

Figure 2: Effect of PKC activation on Ionomycin-modulated cytosolic Ca^{2+} levels of HMC-1^{560,816} cells. Cytosolic Ca^{2+} profile: **(A)** PMA 100 ng/ml pre-treated cells after addition of Ionomycin 0.1 µM, **(B)** addition of Ionomycin 1 µM, **(C)** addition of Ionomycin 10 µM. Ca^{2+} 1 mM was restored to the extracellular medium. **(D)** Intracellular Ca^{2+} reservoir depletion in cells pre-treated with and without PMA 100 ng/ml after addition of different concentrations of Ionomycin. Mean+/-SEM of four experiments. (*) Significant differences between Ionomycin and PMA pre-treated cells.

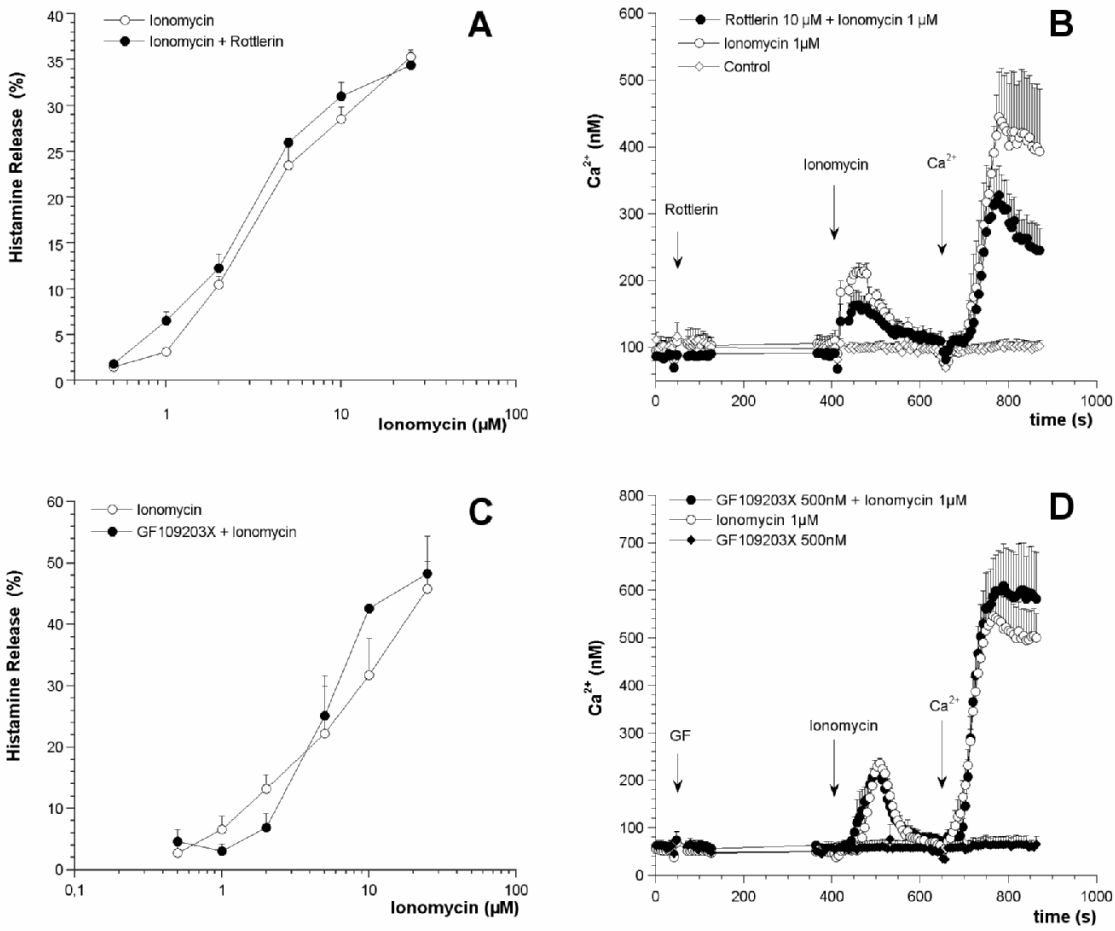

Figure 3: Effect of PKC inhibition on histamine release and cytosolic Ca^{2+} levels of HMC-$1^{560,816}$ cells. Histamine release profile of HMC-$1^{560,816}$ cells: Pre-incubation during 10 minutes with **(A)** Rottlerin 10 µM or **(C)** GF109203X 500 nM and afterwards stimulated with different concentrations of Ionomycin for 10 minutes at 37°C. Cytosolic Ca^{2+} profile of HMC-$1^{560,816}$ cells: **(B)** Rottlerin 10 µM or **(D)** GF109203X 500 nM pre-treated cells after addition of Ionomycin 1 µM. Mean+/-SEM of three experiments.

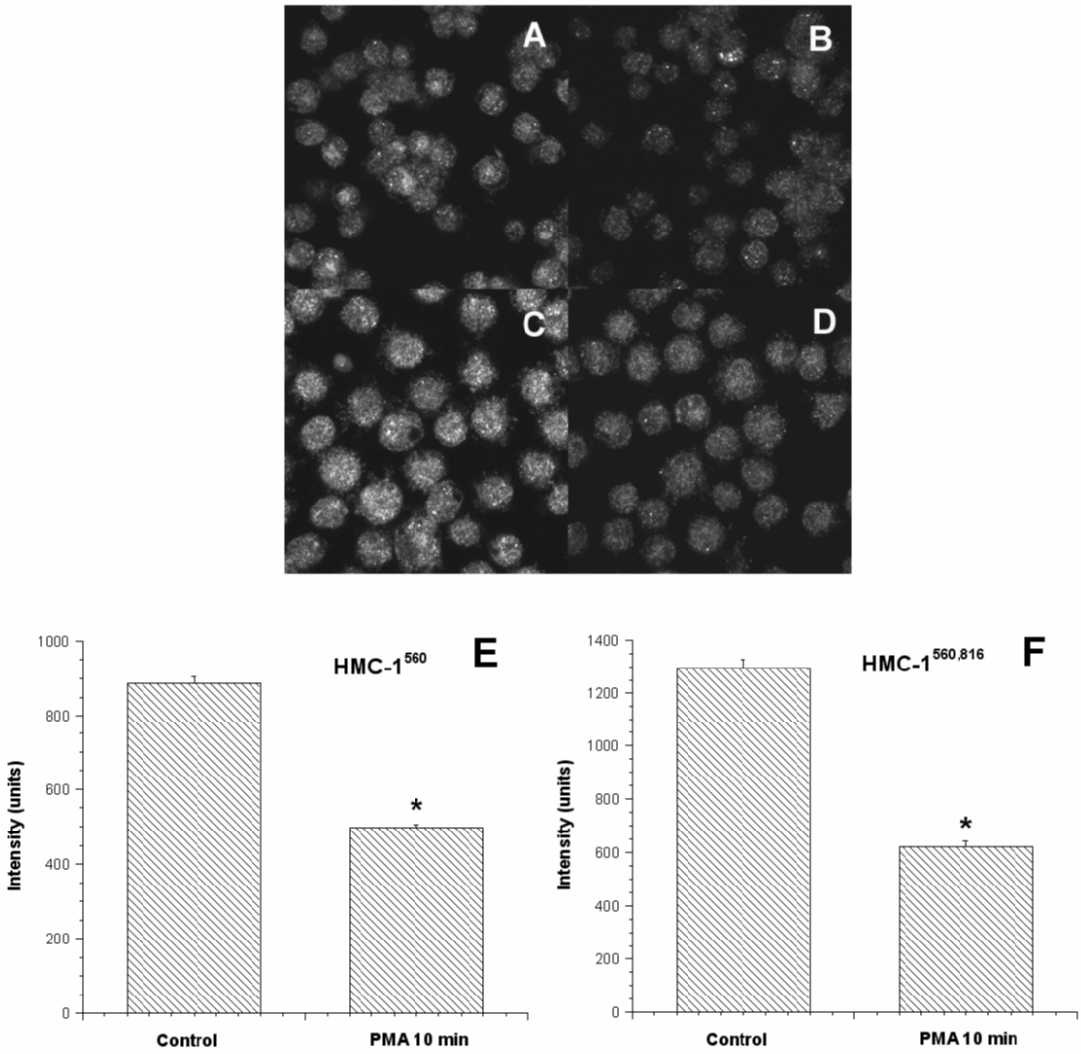

Figure 4: Effect of PKC activation on PKC δ translocation in HMC-1^{560} and HMC-1^{560,816} cells. Cells were stimulated with PMA (100 ng/ml) for 10 minutes in saline solution at 37°C and observed in the confocal microscope after immunocytochemistry: HMC-1^{560} **(A)** control cells and **(B)** PMA-treated cells, HMC-1^{560,816} **(C)** control cells and **(D)** PMA-treated cells. Results for fluorescence intensity of **(E)** HMC-1^{560} cells and **(F)** HMC-1^{560,816} cells. Mean+/-SEM of three experiments.

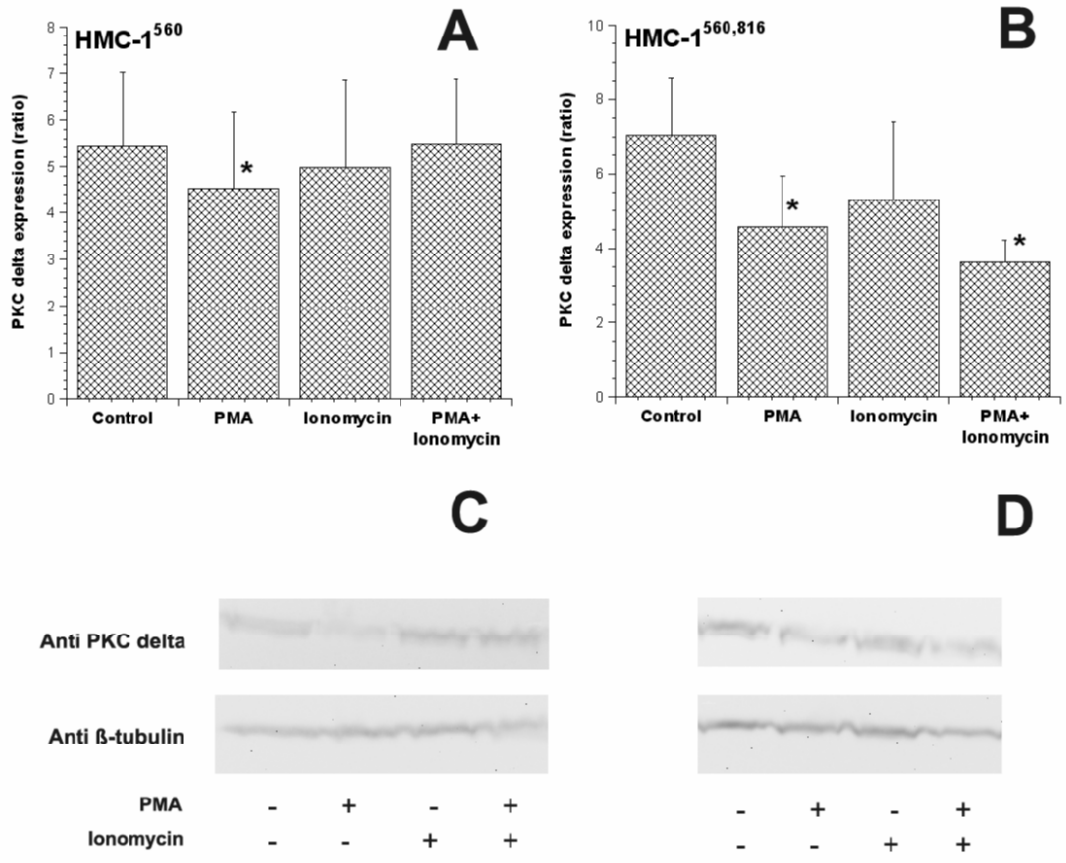

Figure 5: Effect of PKC activation and Ca^{2+} mobilisation on cytosolic PKC δ expression in HMC-1^{560} and HMC-$1^{560,816}$ cells. Cytosolic PKC δ expression of **(A)** HMC-1^{560} and **(B)** HMC-$1^{560,816}$ cells after 10 minutes of pre-incubation with PMA 100 ng/ml and afterwards stimulated with Ionomycin 1µM for 10 minutes at 37°C. Western blot of one representative experiment of three from **(C)** HMC-1^{560} and **(D)** HMC-$1^{560,816}$ cells. (*) Significant differences between control and treated cells. Mean+/-SEM of three experiments.

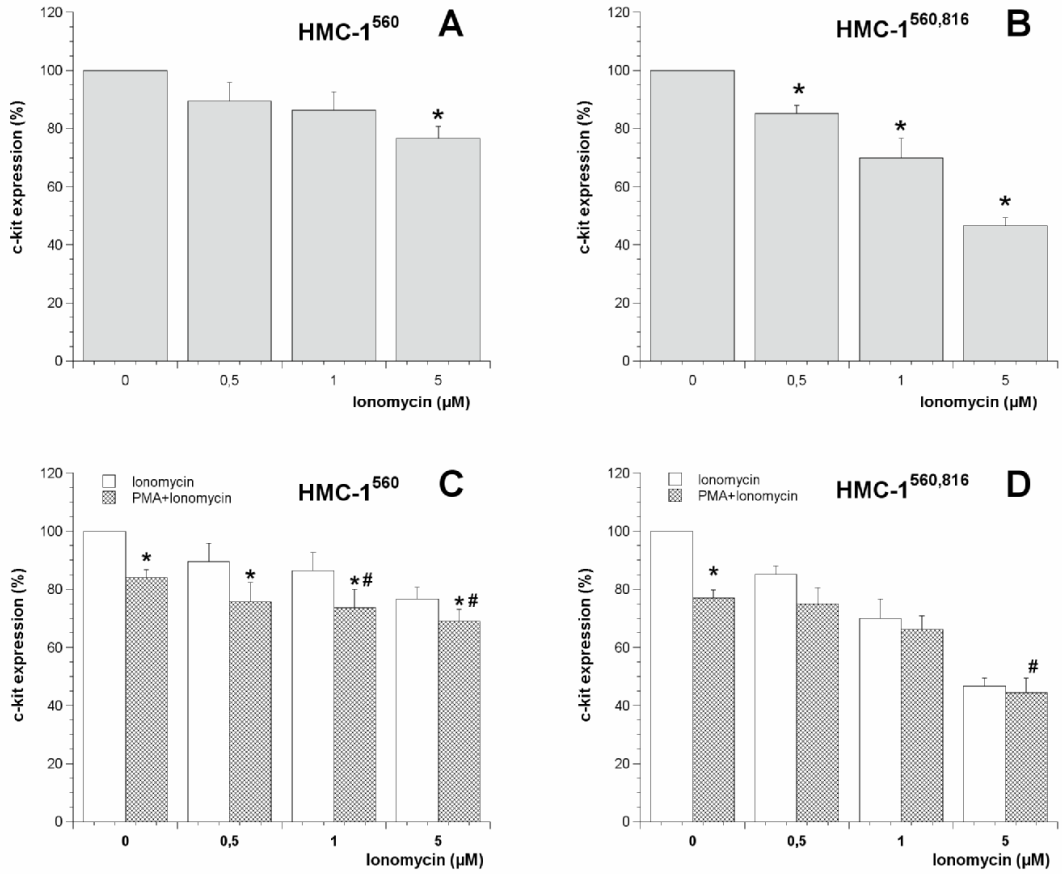

Figure 6: Effect of PKC activation and Ca^{2+} mobilisation on c-kit expression in HMC-1^{560} and HMC-$1^{560,816}$ cells. (A) HMC-1^{560} and **(B)** HMC-$1^{560,816}$ cells after stimulation with different concentrations of Ionomycin for 10 minutes in saline solution at 37°C. (*) Significant differences between control and Ionomycin treated cells. **(C)** HMC-1^{560} and **(D)** HMC-$1^{560,816}$ cells after pre-incubation during 10 minutes with PMA 100 ng/ml and afterwards stimulated with different concentrations of Ionomycin for 10 minutes at 37°C. (*) Significant differences between Ionomycin and PMA/Ionomycin treated cells. (#) Significant differences between PMA and PMA/Ionomycin treated cells. C-kit expression was observed by flow cytometry. Mean+/-SEM of three experiments.

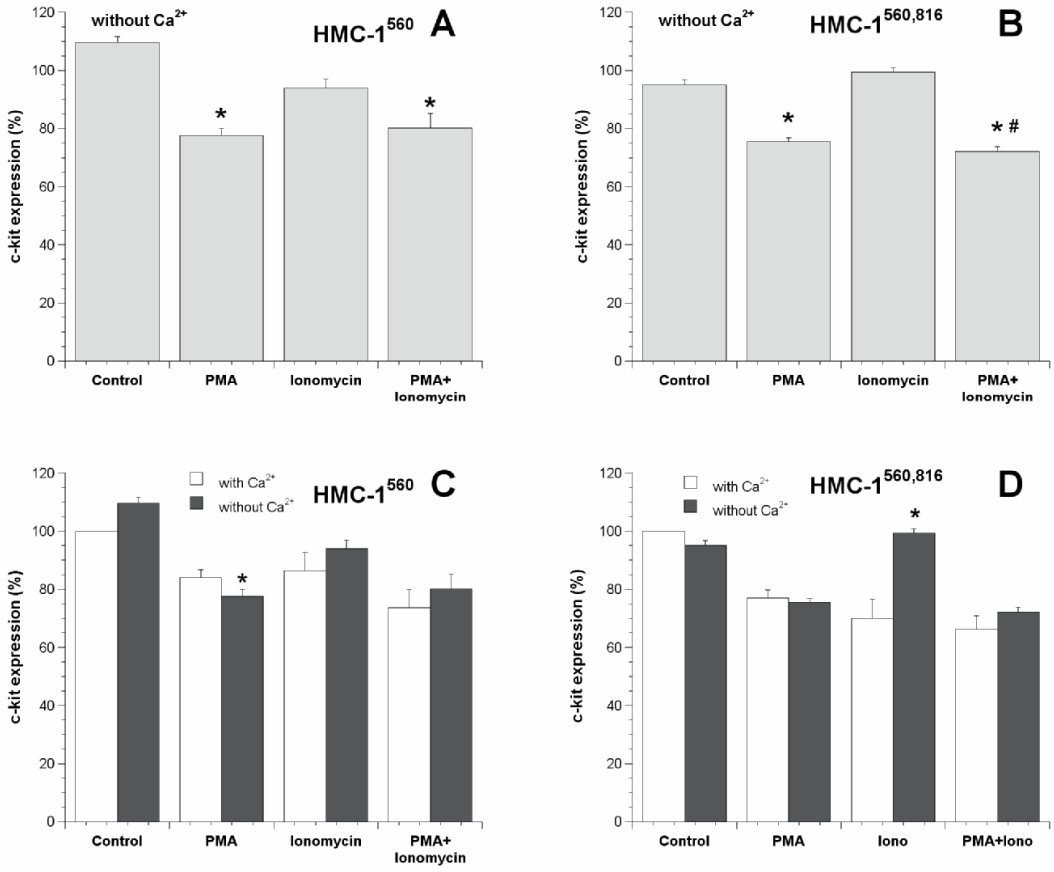

Figure 7: Effect of the absence of extracellular Ca^{2+} on PMA and Ionomycin-induced c-kit down-regulation in HMC-1^{560} and HMC-$1^{560,816}$ cells. (A) HMC-1^{560} and **(B)** HMC-$1^{560,816}$ cells after pre-incubation with PMA 100 ng/ml during 10 minutes in saline solution without Ca^{2+} and afterwards stimulated with Ionomycin 1µM for 10 minutes at 37°C. (*) Significant differences between control and treated cells. (#) Significant differences between Ionomycin and PMA/Ionomycin treated cells. C-kit expression of **(C)** HMC-1^{560} and **(D)** HMC-$1^{560,816}$ cells in the presence and absence of extracellular Ca^{2+}. (*) Significant differences between cells in Ca^{2+} containing and Ca^{2+} free medium. C-kit expression was observed by flow cytometry. Mean+/-SEM of three experiments.

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3.4 RESUMEN

SECCIÓN I

I.A:

Influence of the Tyrosine Kinase Inhibitors STI571 (Glivec®), Lavendustin A and Genistein on Human Mast Cell Line (HMC-1^{560}) Activation

Influecia de los inhibidores de cinasas de tirosina STI571 (Glivec®), lavendustina A y genisteina en la activación de la línea celular de mastocitos humanos HMC-1^{560}

En este trabajo se ha utilizado la línea celular de mastocitos humanos HMC-1^{560} para estudiar los efectos de la inhibición de TyrKs en la liberación de histamina como consecuencia de cambios intracelulares de Ca^{2+} o del pH. Este estudio es importante porque se ha demonstrado que el inhibidor de la TyrK de c-kit STI571 inhibe la proliferación e induce apoptosis en mastocitos con el c-kit salvaje y aún más en células con la mutación 560.

Los inhibidores de TyrKs STI571, lavendustina A y genisteina disminuyen la liberación espontánea en histamina de células HMC-1^{560} después de 24 horas de incubación con uno de los compuestos. Se comparan los resultados con los del estabilizador de membranas celulares de mastocitos, cromoglicato de sodio. Además la incubación de células HMC-1^{560} a tiempos largos con STI571 aumenta la exocitosis inducida por una alcalinización. Se obtiene una liberación de histamina aún más alta si la PKC de los mastocitos pre-tratados con STI571 ha sido activada anteriormente a la alcalinización. No se observan cambios en la desgranulación si la PKC está inhibida por rottlerina, GF109203 or Gö6976. Lavendustina A y genisteina tienen efectos muy similares a los

de STI571 depues de 24 horas de incubación, mientras que el comoglicato no afecta la liberación de histamina inducida por una alcalinización intracelular. Los inhibidores de la TyrK no modifican la exocitosis estimulada con el ionóforo de Ca^{2+} ionomicina. Los resultados obtenidos con células HMC-1^{560} tras 24 horas de incubación con STI571 muestran que el pH_i se alcaliniza aún más en células pre-tratadas con el inhibidor y estimuladas después con NH_4Cl, mientras que los niveles del Ca^{2+} citosólico no cambian durante todo el experimento. No se observan diferencias ni en cambios de pH_i ni de Ca^{2+} citósolico entre células incubadas con o sin STI571 y estimuladas después con ionomicina.

Este trabajo indica otra vez la importancía de pH_i como señal celular y apunta que STI571 tiene rutas de transduccion en común con otros inhibitores de TyrKs.

I.B:

STI571 (Glivec®) Affects Histamine Release and Intracellular pH After Alkalinisation in HMC-$1^{560, 816}$

STI571 afecta a la liberación de histamina y al pH intracelular de HMC-$1^{560,816}$

En este trabajo se ha utilizado la línea celular de mastocitos humanos HMC-$1^{560,816}$ para estudiar los efectos del inhibidor de la TyrK de c-kit STI571 sobre la liberación de histamina y sobre los niveles intracelulares de Ca^{2+} y de pH. Se ha demonstrado que el compuesto inhibe la proliferación e induce apoptosis en las células HMC-1^{560}, mientras que no tiene estos efectos en la línea HMC-$1^{560,816}$.

La exocitosis de las células HMC-$1^{560,816}$ se puede estimular con una alcalinización inducida por NH_4Cl o con un aumento en el Ca^{2+} citosólico inducido por ionomicina. Además, la pre-incubación durante 24 horas con STI571 reduce la liberación espontánea de histamina de estas

células. En cambio este tratamiento incrementa la respuesta celular tras alcalinización, pero no afecta a la respuesta inducida por ionomicina. Las células tratadas con el inhibidor muestran un aumento en el pH_i más alto tras añadir el NH_4Cl, mientras que no hay cambios en los niveles de Ca^{2+} citosólico. Además, la activación de la PKC incrementa la liberación de histamina en células HMC-$1^{560,816}$ tratadas con STI571 y estimuladas después con NH_4Cl. Sorprendentemente la inhibición de la PKC con rottlerina tiene los mismos efectos. Se puede observar que en estas células el pH_i aumenta aún más tras la alcalinización que en las células control.

En este estudio se muestra por primera vez los efectos de STI571 en células con la mutación en la posición 816 del proto-oncogeno de c-kit. Los resultados indican que las señales implicadas en la proliferación y en la exocitosis están moduladas por diferentes rutas de transducción. STI571 no solamente inhibe la TyrK de c-kit, sino también influye en el pH_i tras una alcalinización y esto induce liberación de histamina en ambas sublíneas HMC-1.

Este trabajo es importante porque el 80% de los pacientes con SM tiene mastocitos con la mutación 816 y un mejor conocimiento de las rutas de transducción involucradas en la activación de estas células puede facilitar el desarollo de nuevos medicamentos.

SECCIÓN II

II.A:

Role of Extracellular HCO_3^- in Cytosolic pH Regulation and Cell Viability of HMC-1 Human Mast Cells

Papel de HCO_3^- extracelular en la regulación del pH citosólico y de la viabilidad celular de los mastocitos humanos HMC-1

Estudios anteriores han demonstrado la importancia del pH_i en la activación de las células HMC-1^{560} y HMC-$1^{560,816}$. En este trabajo se estudia el papel de Na^+ y HCO_3^- extracelular en la regulación del pH_i en estas células. Se comprueba la participación de transportadores de HCO_3^- y de intercambiadores de Na^+/H^+ en el mantenimiento del pH_i basal y las consecuencias en la viabilidad celular de deficiencias en este processo.

HCO_3^- y CO_2 forman parte del mecanismo principal implicado en la regulación del pH_i en células humanas. El pH intracelular de células HMC-1 cae inmediadamente en un medio extracelular sin HCO_3^-. La caída es aún más evidente en ausencia de Na^+ y HCO_3^-. Además se observa que el descenso en el pH_i se estabiliza al cambiar el tampón extracelular por uno más alcalinizado o al añadir diferentes concentraciones de $NaHCO_3$. En cambio, la adición de $KHCO_3$ no frena la caída. En las células HMC-$1^{560,816}$ el inhibidor de las anhidrasas de carbono, acetazolamida, retarda la baja de pH_i, mientras que inhibidores de intercambiadores de aniones y de NHE la potencian.

En medios sin HCO_3^- o sin Na^+/HCO_3^- extracelular una incubación a tiempos cortos disminuye significativamente la viabilidad de células HMC-$1^{560,816}$, y también, pero menos significativo, la de las células HMC-1^{560}. El tratamiento durante 24 horas con diferentes inhibidores de

intercambiadores anionicos disminuye la viabilidad de ambas sublíneas HMC-1.

Estos resultados demuestran la existencia de un mecanismo regulatorio del pH_i dependiente de Na^+ y HCO_3^- y además conectado a NHE en las células HMC-1. Tanto el HCO_3^- extracelular como el funcionamiento de un intercambio de estos aniones son importantes para la viabilidad celular.

II.B:

NH_4Cl Induced Alkalinisation and Exocytosis of HMC-1 Human Mast Cells are Regulated by NHE1 and a Na^+/HCO_3^- Exchanger

La alcalinización y la exocitosis inducidas por NH_4Cl en los mastocitos humanos HMC-1 están reguladas por NHE1 y un intercambiador de Na^+/HCO_3^-

Estudios anteriores han demonstrado que una alcalinización por si sola desencadena el proceso que conduce a la desgranulación de las células HMC-1^{560} y HMC-$1^{560,816}$. En este trabajo se estudia el papel de Na^+ y HCO_3^- extracelular en la alcalinización inducida por NH_4Cl y en la liberación de histamina provocado por ella.

La ausencia de HCO_3^- extracelular disminuye de forma significativo la alcalinización inducida por NH_4Cl. Esta disminución es aún más notable si a parte de HCO_3^- también el Na^+ es sustituido. La diferencia es más marcada en células HMC-1^{560} que en HMC-$1^{560,816}$. En un medio extracelular sin Na^+ y HCO_3^- la células HMC-1^{560} dejan de liberar histamina, mientras que las HMC-$1^{560,816}$ solo disminuyen la liberación del mediador en las mismas condiciones. Utilizando diferentes inhibidores del intercambio anionico en medio salino fisiológico no se observan efectos, ni en el cambio del pH_i, ni en la liberación de histamina tras la alcalinización con NH_4Cl. Además, experimentos hechos en ausencia de Cl^- extracelular demuestran que no afecta a la exocitosis o a la

alcalinización. En cambio la inhibición de NHE inhibe la subida del pH_i al añadir NH_4Cl, pero no modifica la liberación de histamina.

Este studio demuestra que la alcalinización y la exocitosis en las células HMC-1 inducidas por NH_4Cl son dependientes de la presencia de Na^+ y HCO_3^- extracelular y de NHE, pero son independientes de Cl^- extracelular.

SECCIÓN III

III.A:

PKC stimulation has different effects in human mast cells HMC-1^{560} and HMC-$1^{560,816}$

La estimulación de la PKC tiene diferentes efectos en los mastocitos humanos HMC-1^{560} y HMC-$1^{560,816}$

En este trabajo se ha utilizado las líneas de mastocitos humanos HMC-1^{560} y HMC-$1^{560,816}$ para estudiar los efectos de la estimulación de PKC y/o del tratamiento con un ionóforo de Ca^{2+} sobre la liberación de histamina, el Ca^{2+} citosólico y la expresión de c-kit.

La estimulación de células HMC-$1^{560,816}$ con el ionóforo de Ca^{2+} ionomicina provoca exocitosis y este efecto está inhibido por la estimulación de PKC δ. Sin embargo la inhibición de PKC no modifica la respuesta celular. La liberación de histamina inducida por ionomicina es dependiente de la presencia de Ca^{2+} en el medio extracelular, aunque el Ca^{2+} citosólico aumenta al vaciar los reservorios intracelulares.

Dado que se han observado diferentes efectos en la liberación de histamina producida por estos estimulos en las células HMC-1^{560} y HMC-$1^{560,816}$, se realizan estudios sobre la expresión de c-kit. La ionomicina

reduce la expresión de c-kit de forma dependiente de la dosis utilizada en las dos sublíneas HMC-1. Los resultados estan de acuerdo con el incremento en el Ca^{2+} citosólico provocado por ionomicina. El efecto está completamente abolido en un medio extracelular sin Ca^{2+} en las células HMC-$1^{560,816}$, pero no en las HMC-1^{560}. Una estimulación anterior de la PKC aumenta la respuesta inducida por el ionóforo en las células HMC-1^{560}, pero no en las HMC-$1^{560,816}$.

De este trabajo se concluye que en la línea celular HMC-1, PKC δ actúa como un regulador negativo en la desgranulación inducida por ionomicina. PKC e ionomicina regulan la disminución de la expresión de c-kit por rutas de transducción completamente diferentes en las células HMC-1^{560} y HMC-$1^{560,816}$. Las diferencias pueden ser debidas a la activación de distintas cinasas. No hay pruebas de una conexión de la liberación de histamina y la disminución de c-kit inducidas por la estimulación PKC/Ca^{2+}.

4. CONCLUSIONES

1.- El fármaco STI571 reduce la liberación espontánea de histamina en células HMC-1^{560} y HMC-$1^{560,816}$ a través de cinasas de tirosina diferentes de las que se activan por el receptor c-kit.

2.- El fármaco STI571 aumenta la liberación de histamina inducida por una alcalinización en células HMC-1^{560} y HMC-$1^{560,816}$.

3.- Las células HMC-1^{560} y HMC-$1^{560,816}$ expresan el intercambiador NHE1 y un transportador de HCO_3^- dependiente de Na^+ que es más sensible en las células con dos mutaciones.

4.- El transportador de HCO_3^- y la presencia de este ión en el medio extracelular son imprescindibles para la supervivencia de las células HMC-1^{560} y HMC-$1^{560,816}$.

5.- El transportador de HCO_3^- y el intercambiador NHE participan en la alcalinización citosólica en las células HMC-1^{560} y HMC-$1^{560,816}$.

6.- En las células HMC-1^{560} y HMC-$1^{560,816}$, la PKC δ es un regulador negativo de la liberación de histamina inducida por un aumento del Ca^{2+} citosólico.

7.- La activación de la PKC o un aumento en el Ca^{2+} citosólico reduce por distintos mecanismos la expresión del receptor c-kit en las células HMC-1^{560} y HMC-$1^{560,816}$. Estas rutas son distintas de las que inducen liberación de histamina.

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